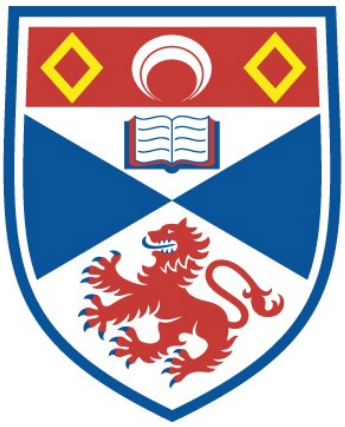
SOME ASPECTS OF EARLY GAMETOGENESIS IN AMPHIBIA

Michael Henry Lloyd Snow

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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SOME ASPECTS OF EARLY GAMETOGENESIS IN AMPHIBIA.

by

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Thesis submitted for the degree of Doctor of Philosophy.

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DECLARATION

I hereby declare that this Thesis is based upon the results of my own work and has been written by me without assistance. Professor H.G. Callan and I are jointly responsible for the publication of a paper, a reprint of which can be found inside the front cover of the Thesis. It is included for reference purposes and my contribution to the publication is not reproduced in this Thesis.

My Thesis has not been submitted for any other degree.

MICHAEL HENRY LLOYD SNOW.

CERTIFICATE

I certify that Mr. Michael Henry Lloyd Snow has spent nine terms at an investigation of Some Aspects of early Gametogenesis in Amphibia, that he has fulfilled the conditions of Ordinance General No. 12, and that he is qualified to submit the accompanying thesis for the degree of Doctor of Philosophy.

H.G. CALLAN,
Professor of Zoology.

UNIVERSITY CAREER

I first matriculated in the University of St. Andrews in October 1962, and graduated with an Honours B.Sc. (Class II i) in Zoology in June 1966. Since September 1966 I have continued the investigation of lampbrush chromosomes that I started in my final undergraduate year and also extended my studies to include some aspects of male meiosis in Amphibia. The results of my researches are presented in this Thesis for the degree of Doctor of Philosophy.

I N D E X

GENERAL INTRODUCTION

Following the observations of Mirsky & Ris (1951) many reports have confirmed that there is a great range in the DNA content of the gamete nuclei (the c-value according to Hewson Swift) of diploid chromosomal organisms (see Callan, 1967; Goin, Goin & Bachmann, 1968; and Whitehouse, 1968). The large differences in c-value that exist between distantly related organisms do not simply reflect organizational complexity. Mammals, e.g. man, rat and horse with a c-value of around 3 μμg are assuredly more complex than Urodeles, which show c-values ranging from 10 μμg in *Desmognathus monticola* to 80 μμg in *Amphiuma*, and fish such as trout and carp with c-values of around 2 μμg. Callan (1967) also cites examples of large differences between organisms related at the genus level, e.g. *Gammarus pulex* spermatids contains 3 times as much DNA as those of *G.chevreuxi* and spermatids of the planarian *Mesostoma ehrenbergi* contain 11 times as much DNA as those of *M.lingua*. Callan has indicated that in many of these organisms simple explanations based upon chromosome number do not apply, and goes further to reject convincingly explanations based upon multistranded chromosomes.

To be consistent with the evidence of the genetical phenomena of mutation and recombination and the cytological data concerning the

semi-conservative mechanism of DNA replication (Taylor, Woods & Hughes, 1957); the frequencies of 'single' and 'twin' sister chromatid exchanges (Taylor, 1958); the kinetics of DNase breakage of newt lampbrush chromosomes (Gall, 1963b) taken in conjunction with the ultrastructural data provided by Miller (1964) on lampbrush chromosome loop axis and main chromosomal DNA axis thickness, chromosome models based upon long single strands of DNA must be considered. Considerable evidence has accumulated over the past few years that suggests chromosomal DNA consists, at least in part, of serially repeated sequences. Keyl (1964, 1965a and 1965b) presents cytological evidence for such a serial duplication within chromatids. Keyl showed that the DNA contents of homologous bands of the salivary gland chromosomes in hybrids of Chironomus thummi thummi x Ch.th.piger may differ in the ratio of 1 to 2,4,8 or 16. Since Keyl (1965b) found that the salivary gland chromosome and the gametic DNA values of thummi are both 27% higher than the corresponding values for piger he argued against the possibility of unequal polyteny being the explanation for these ratios. He similarly dismisses the possibility that the different values arose by unequal crossing over during meiosis since the ratios 1:3, 1:5 etc. were never observed, and suggests that the geometric increase in DNA content of these chromosome bands arose

as the result of serial duplication of these specific replicating units. At the biochemical level Britten & Kohne (1965) and Walker (1968) have indicated that considerable repetition of nucleotide sequences may exist within the DNA of a single species. The phenomenon of genomic repetition is a well documented feature of the nucleolus organizer locus in many animals (Ritossa and Spiegelman, 1965; Wallace and Birnstiel, 1966; Brown and Weber, 1968; and Gall, 1969).

Callan and Lloyd (1960) were the first to suggest that 'genes' might be serially repeated. The hypothesis was put forward as a possible explanation for some of their observations made on the lampbrush chromosomes of Triturus cristatus. They had observed that the lateral loops of these chromosomes exhibited an asymmetry of constant polarity and that the loops apparently grew in length at the expense of chromomeric DNA. They suggest that these phenomena can be explained if the lateral loop axis DNA issues from one side of a chromomere and is retracted into the other side. Callan and Lloyd were also impressed by specific loci where the lateral loop ribonucleoprotein matrix was the distinctive feature of the loops. Since the dimensions of the lateral loops would indicate that many genes exist along each

lateral loop, the unusual but constant, nature of the loop matrix at these loci suggested to Callan and Lloyd that the genes in these loops must be similar, perhaps identical. They therefore postulated that there may exist in each lateral loop a series of repeated genes consisting of a master copy, which alone is involved in recombination and a number of slave genes. This theory is supported in part by the observation that chiasmata are not formed within the lengths of loops but always involve the chromomeres or interchromomeric DNA of the lampbrush chromosomes, which suggests that the chromomere behaves as a unit with regard to recombination. More significant are the observations that lateral loops of characteristic morphology show classical Mendelian inheritance in F_1 and backcross hybrids between the subspecies of T.cristatus and that intrachromosomal recombination between loops has been demonstrated as an outcome of meiosis in F_1 female hybrids.

Any model for such chromosome organization requires that 'slave' genes can be matched with the master copy at some stage in development. This arises because genetic data demands that the homogeneity of each gene product is maintained. Mutational changes in any particular allele are all-or-none effects, a characteristic it is difficult to envisage if each individual of a family of genes were free to mutate and then express itself independently. Callan (1967) has proposed a model whereby this matching might be achieved.

In his model Callan proposes a sequence of events whereby a linear sequence of genes can be corrected, individually, against a master gene which is situated at one end of the sequence. No breakage of either polynucleotide chain is postulated and the process results in the polarized production of a loop of DNA comprising 'corrected' slaves. Such a process could account for the structure of the lampbrush chromosomes found in the primary oocytes of many animals.

Although attempts have been made to demonstrate such a polarized movement of DNA in the lateral loops of lampbrush chromosomes the results of the investigations have been inconclusive. Gall and Callan (1962), and Gall (1963a) report single, specific pairs of lateral loops in the lampbrush chromosomes of T.cristatus cristatus and T.viridescens in which RNA synthesis, as judged by H^3-uridine incorporation, is confined to a short region at the thinner end of these asymmetrical loops. Gall & Callan further observed that in T.c.cristatus these loops are labelled over about half their length after a 4 day availability of H^3-uridine and are fully labelled after some 10 days. Such sequential labelling can only be explained in terms of polarized movement but unfortunately does not distinguish between movement of lateral loop matrix around a stationary loop axis and movement of matrix and axis simultaneously.

Despite the close similarities between these two reports the loops concerned must be regarded as the exception rather than the rule. The vast majority of the pairs of lateral loops are similar in appearance and any one pair is not readily and easily distinguishable from its immediate neighbours. All these loops show uniform incorporation of H^3-uridine throughout their entire length and the only suggestion of polarized movement in them is their morphological asymmetry. For the purposes of repeated observations it is essential that particular loops for study should be easily located and recognized and should invariably be present at a convenient stage of oocyte development. It is for these reasons that studies on lampbrush chromosome loops have largely been confined to those 'marker' loops distinguishable by their size, morphology or by the nature of their matrix. The best documented lampbrush chromosome 'maps' are those of Callan and Lloyd (1960) for the four subspecies of the newt T.cristatus, and consequently much of the lampbrush chromosome data gathered in the last few years has been derived from these newts. The work on lampbrush chromosomes described below is derived from the subspecies T.c.cristatus.

Snow and Callan (1969) have described a different approach to the problem of polarized loop movement whereby Actinomycin D was used in vivo as an inhibitor of RNA synthesis and as a result of which the de novo

growth of lateral loops was observed for the first time. In the particular case of the giant granular loops of chromosome XII evidence was found which lends considerable support for the hypothesis that lateral loop axis and matrix move simultaneously. However, as it is this pair of lateral loops which exhibits the sequential labelling with H^3-uridine this additional data serves perhaps only to accentuate the peculiar nature of these loops. Possibly of greater significance to generalized chromosome physiology are two other phenomena described by Snow and Callan. First, the demonstration of discontinuities in labelling pattern in some of the normal lateral loops during the post Actinomycin D recovery period which are also most easily explained in terms of simultaneous matrix and axis movement, and secondly the demonstration that under certain physiological conditions (i.e. dilute saline) the giant granular loops can be made to look like normal lateral loops, albeit very large ones. This second observation suggests that the major difference between normal lateral loops and the giant granular loops lies in the composition of their ribo-nucleo protein matrix and that the mechanics involved in loop axis extension and the maintenance of loop length are similar if not identical for all lateral loops. More detailed discussion of these and related aspects of lampbrush chromosome organization can be found in Snow and Callan (1969).

It has already been remarked that lateral loops appear to grow in length at the expense of chromomeric DNA because short loops are associated with large chromomeres and vice versa. Should this interpretation be correct a profitable line of investigation might be to interrupt or upset in some way the equilibrium between extension and retraction that must exist if polarized axis movement is to maintain a loop of constant length. Acceleration of extension but not retraction should produce loops of steadily increasing length and acceleration of the retraction process alone would have the opposite effect. In this approach it is essential that the design of the experiment allows effects on a non-polarized loop extension to be distinguished from those on a polarized system. In other words one must be sure that loops of changing length are due to loop axis being added or removed at one end only.

RNA is actively synthesized on the lateral loops of lampbrush chromosomes and the suppression of RNA synthesis has been shown to result in loss of loop components from these chromosomes (Izawa, Allfrey & Mirsky, 1963, and Snow and Callan, 1969). It seems reasonable therefore to assume that the existence of lateral loops is a consequence of RNA synthesis rather than the other way round, and further that variation in the rate of RNA synthesis will result in corresponding

variations either in the overall length of extended lateral loop axis or in the rate at which the DNA axis is exposed for transcription. This second alternative does not necessarily mean changes in the rate of axis extension, it may simply reflect an acceleration in the rate of transcription itself. Gall and Callan (1962) and Macgregor (1963) have observed the effects of gonadotrophin and of hypophysectomy on the physiological state of lampbrush chromosomes. Gonadotrophin increases the rate of RNA synthesis and of labelled phosphate uptake while hypophysectomy reduces phosphate uptake. Although morphological changes were observed in some loops - neither of these treatments invariably changes the length of lateral loops. Unless changes in transcription rate are the sole factor in the differing rates of RNA synthesis this observation is open to only one interpretation, i.e. that variations in the rate of RNA synthesis are reflected in changes in axis extension, but that there is an equilibrium between extension and retraction that is not upset by changes in rate of RNA synthesis. This inference is explicable only in terms of Callan's theory of polarized extension and retraction of lateral loop axis but clearly before a definite conclusion can be drawn these two processes must be experimentally separated. In this respect the extreme case, where one process exists in the absence of the other represents the ideal situation and by the use of Actinomycin D in conjunction with

gonadotrophin or with mutagens causing chromosome breakage it was hoped to achieve this ideal. The results of these investigations are reported in Part 1 of this thesis.

During my work with lampbrush chromosomes my interest was aroused by certain changes that were induced in the nuclear sap and particularly in the peripheral nucleoli of these newt oocytes, under the influence of Actinomycin D. As a result my studies were extended to include light and electron microscope studies of some non-chromosomal nuclear inclusions during and after the standard treatment with Actinomycin D. This work is also described in Part 1 of this thesis.

In Part II I have reported the results of an investigation of a different aspect of chromosome organization. The presence in the genome of many, if not all, higher organisms of large portions of DNA composed of many repeated nucleotide sequences poses considerable problems with respect to genetic mutation and recombination in which these 'families' of genes behave as single units. Certainly in the case of multiple ribosomal genes and probably in many of the other gene families (Britten, 1969) the DNA is transcribed and these reiterated nucleotide sequences must therefore be regarded as genetically active. It is also clear that these reiterated sequences have been maintained over a considerable evolutionary period, although the conflict between Britten and Kohne, who believe the sequences to

have a recent origin and to be in the process of diverging, and Walker, Flamm and McCallum (1969) who believe them to be conserved and probably to already have a functional role, is far from being resolved. In the case of ribosomal cistrons the demand for gene product has been met in a variety of ways, for the special case of oocyte production, viz: in Amphibia developing oocytes may contain several thousand free nucleoli, each containing its own ribosomal DNA e.g. Triturus (Macgregor, 1965), or even multiple nuclei e.g. Ascaphus (Macgregor & Kezer, 1970). In Arthropods the RNA (principally ribosomal) synthesized by a number of nurse cells is transported via a system of microtubules into the developing oocyte (Macgregor & Stebbings, 1970). It seems to me reasonable to suppose that each of these systems has been selectively established and to confer advantage on the organism concerned. The recent demonstration by Jones (1970) and Gall and Pardue (1970) that mouse satellite DNA (a highly reiterated sequence) is localized around the centromeres of the chromosomes suggests a possible functional role for this DNA, in which case this sequence also has probably been preserved.

Should such preservations be the case for all reiterated sequences a mechanism whereby significant mutational errors can be avoided or removed becomes essential. Removal or correction is probably simpler and the model for such a process described by Callan (1967) would operate

immediately after recombination, presumably during synapsis or pachytene of meiosis. As mentioned above Callan suggested that the lampbrush phase of oocyte meiosis may be the manifestation of such a correction process. It must however also occur during spermatogenesis so I turned my attention to male meiosis in search of evidence for such a correction process there. It was not my aim to show the presence of a lampbrush phenomena during male meiosis (Hess and Meyer, 1963; Meyer and Hess, 1965, have already demonstrated this in Drosophila hydei and D.neohydei) but to look for differences in time course of meiotic prophase in related species which could be related to their nuclear DNA content. Hopefully there would be some indication that the size of the families of reiterated sequences differed proportionately and was reflected by differences in the duration of the correction process.

Several important arguments need to be considered before it can be assumed that such a difference might exist between male meioses. The first and most fundamental is that differences in DNA content between related species are not explicable simply in terms of complexity of the respective organisms. There can be little doubt that this cannot be the only explanation where the differences in c-value are great, as for example in the amphibia, where, according to Goin, Goin and Bachmann(1968) Anuran c-values range from about 1.5 μμg in

Scaphiopus h. holbrookii to about 9 μμg in Rana catesbiana and Urodele values from 10 (Desmognathus monticola) to about 80 μμg in Amphiuma. It is unreasonable to thing of 6- to 9-fold differences in complexity, however that may be defined, within each of these two groups and even more unlikely that Amphiuma requires 50-60 times more different genes than Scaphiopus. The second consideration is whether or not the families of reiterated sequences can be expected to behave as single units with regard to the postulated correction process and consequently whether correction is a sequential process taking place from one end of the series to the other. It has been stated earlier that Keyl's data (Keyl, 1964, 1965a and b) make it clear that a series of duplications is the most likely method for production of large families of sequences, but it poses the question of what happens to the duplicate 'master' gene each time? Either subsequent copies of masters behave as slaves, or only the slaves are duplicated each time. This question becomes significant when initiation sites for replication and, especially, correction, are considered in relation to replicon size and chromosomal sub-units. Keyl's data is based upon observed differences in DNA content in homologous bands in salivary gland chromosomes, bands which are composed of precisely aligned homologous chromomeres. Pelling (1966)

and Whitehouse (1969) also discuss evidence which clearly implicates the chromomere as the basic chromosomal unit, from the points of view of transcription, replication and recombination. To be consistent with this evidence the chromomere might be expected to behave as a unit in any master/slave correction process. Callan (1963) has stated that the haploid complement of lampbrush chromosomes in T.cristatus exhibits about 5,000 chromomeres with the implication that these may be equated with genes, or at least with families of genes. This equation should not be carried too far. The chromomere is a cytologically defined structure whereas the gene is defined by its effect. Furthermore 5,000 genes seems too few to make a newt and also Callan (1963) demonstrates chromomeres bearing multiple lateral loops implying the genetic subdivision of those chromomeres. Nevertheless Callan's unpublished data on lateral loop inheritance and the data from salivary gland chromosomes (Pelling, 1966) do suggest that chromomeres can, to a great extent, be identified with specific genes.

As mentioned earlier it seems reasonable, in terms of complexity to consider Amphibians as approximately equivalent and therefore to regard Xenopus laevis, Bufo bufo, Triturus vulgaris and Batrachoseps attenuatus as requiring about the same number of genes for their

specification. Since these species show large differences in their c-values, in the approximate ratio of 1:2:10:20 it seemed possible that these differences reflected the sizes of the gene families in these species, and further that the replication and correction of these families entail correlated differences in the duration of certain stages of meiosis.

PART I

Introduction

In the General Introduction I have described one of the major problems concerning chromosome organization and explained how it concerns the particular case of lampbrush chromosomes. In this section I have presented the experimental data that has not been published and in particular attempts to observe the process of lateral loop extension independently of retraction.

I have investigated the possibility that during the *de novo* growth of lateral loops after Actinomycin D treatment the process of extension can be accelerated, as a result of the increase in RNA synthesis following gonadotrophin treatment, in the absence of loop retraction. On the theory of polarized axis movement these conditions are best provided in the special case of the giant granular loop where the persistence of the old loop for several days after treatment (Snow and Callan, 1969) will serve to monitor that retraction of this loop region is not occurring at an accelerated rate. In the case of normal lateral loops it can be assumed that an extension/retraction equilibrium is not established until full loop extension has been achieved. Gonadotrophin treatment in this case might considerably accelerate recovery from antibiotic poisoning.

A second approach to separating extension from retraction has been to create lateral loop breaks *in situ* and after a suitable period of time look for evidence of a continuously extending loop filament. Of the currently available mutagens that produce chromosome breaks the most promising for this investigation was X-irradiation. Miller, Carrier and Von Borstel (1965) report the *in vitro* breakage of lampbrush chromosomes in *T.viridescens* by high doses (2,000 and 5,000 roentgen/min) of X-irradiation. Such doses given *in vivo* lead very quickly to death but Callan, in unpublished

observations, found that newts will survive for long periods after doses of 800 roentgen. Even at this level, however many oocytes degenerate during the 10 days or so immediately following irradiation. In his observations Callan found that lateral loop breaks could be found in chromosomes isolated immediately after irradiation but that on subsequent days there was no evidence for these breaks. Callan concluded that in situ there is a rapid repair of DNA breakage, the continuity of the lateral loops meanwhile being maintained by the nucleoprotein matrix. With the aid of Actinomycin D it was hoped to prevent the loop axis repair and to expose the breakage by stripping off the lateral loop matrix. The results of these investigations are reported below.

The Nucleoli

The oocyte nucleus of T.cristatus contains large numbers of free nucleoli that are not attached to the chromosomes. In oocytes 0.6 - 0.9mm diameter these nucleoli are large spheroidal bodies up to 10μ in diameter and arranged peripherally just inside the nuclear membrane. There may be 1000 such nucleoli in a single germinal vesicle.

The coincidental occurrence of the multi-nucleolate condition with the presence of lampbrush chromosomes led to the belief that the nucleoli of amphibian oocytes were produced in association with the lampbrush phase of oogenesis (Duryee 1941, 1950; Gall 1954 and Miller 1961). There is now, however, compelling evidence that extra-nucleolar production occurs predominantly during early pachytene and only occasionally is there an

increase in nucleolar number in the lampbrush phase. Painter & Taylor (1942) showed that the large number of Feulgen positive granules that appear during pachytene in oocytes of Bufo valliceps migrate to a peripheral position in the nucleus and come to have a nucleolus associated with them during later stages of oogenesis. The discovery that amphibian oocyte nucleoli contain DNA (Miller 1964) has greatly strengthened Painter & Taylor's observation. More recently Gall (1967 and 1968) has found a disproportionately intense synthesis of ribosomal DNA during prophase of the first meiotic division of Xenopus oocytes and this finding has been correlated with the subsequent appearance of peripheral nucleoli (Perkowska, Macgregor & Birnstiel 1968 and Macgregor, 1968).

The role of the nucleolus in cell metabolism has been of especial interest to biologists since the discovery of a lethal anucleolate mutant of Xenopus laevis (Elsdale, Fischberg & Smith, 1958). The discovery of RNA in the nucleolus (Brachet, 1940; and Caspersson & Schultz 1940) had led to the postulate (Brachet, 1952) that the organelle is concerned with cellular RNA metabolism. By 1961 it seemed certain from the autoradiographic data that the nucleolus is the major site of cellular RNA production most of which was known by that time to be ribosomal RNA (see Perry, Hell & Errera 1961). Since that date the accumulation of biochemical data (cf. Birnstiel 1967) has left little doubt that the nucleolus is the cellular site for ribosomal RNA synthesis.

The data presented by Perkowska et al. (1968) for oocytes of X.laevis

indicate that each nucleolus contains approximately 2 x 10^{-14} gm DNA, - a fraction of 1% of the dry weight of a nucleolus. The rest of the nucleolus is composed of RNA and protein. The RNA, which has a base composition similar to that of cytoplasmic RNA (Edström & Gall, 1963; Vincent 1963, and Chipchase & Birnstiel 1963) forms less than 20% of the dry weight, although the actual value varies with the age of the nucleolus and with the measurement techniques employed (Edström, Grampp & Schor 1961, and Finnamore 1961). The remaining 80% or more of the nucleolus is protein (Vincent 1955 and Vincent, Balthus, Lövlie & Mundell 1966).

The morphological arrangement of these components varies according to the cell type. The nucleolar DNA is only occasionally demonstrable by the Feulgen reaction (Painter & Taylor 1942; and Guyénot & Danon 1953) but has been clearly demonstrated in autoradiographs by the specific binding of H^3-Actinomycin D (Ebstein 1967). It is normally eccentrically placed in the Amphibian oocyte nucleolus and lies nearest to the nuclear membrane. The work of Miller (1964) suggests that it exists in ring form. Autoradiography of incorporated H^3-uridine indicates that nucleolar RNA is probably transcribed by this DNA (Lane 1967 & Macgregor 1967). A non uniform distribution of RNA can be demonstrated in nucleoli from Bufo bufo in oocytes stained with gallocyanin chrome alum (see fig. 31).

It has been shown that the non-uniform distribution of nucleolar RNA (due to an eccentric site of synthesis) is reflected in the fine structure of the nucleolus. Electron micrographs indicate quite clearly

that there are two zones in amphibian oocyte nucleoli (Miller 1962, 1966 and Macgregor 1967). These zones have been termed core and cortex and can be readily separated from one another if nucleoli are isolated into dilute veronal or ammonium acetate (Miller 1961 and Macgregor 1965). The core is composed of fibrils 50-100 A^{o} diameter and granules 150-200 A^{o} diameter embedded in an amorphous matrix. The cortex is made up of granules 150-200 A^{o} diameter. Both the fibrils and the granules contain RNA and protein (Marinozzi 1963, 1964; Marinozzi & Bernhard 1963; Bernhard & Granboulan 1963; and Granboulan & Granboulan 1964a, 1964b, 1965). The relative amounts of core and cortex in each nucleolus varies with the age of the oocyte (Miller 1966, and Macgregor 1967).

Incorporation of RNA-precursors into nucleoli is immediately inhibited by Actinomycin D (Reich, Franklin, Shatkin & Tatum 1961; and Izawa, Allfrey & Mirsky 1963a). Indeed, Perry (1962, 1963) found that low concentrations of Actinomycin D suppress nucleolar and cytoplasmic RNA synthesis but not chromosomal RNA synthesis. The inhibition of nucleolar RNA synthesis by Actinomycin D causes a disruption of the normal nucleolar fine structure, the extent of the changes being dependent upon Actinomycin D concentration. These changes have only occasionally been observed by light microscopy (Bierling 1960, Duprat, Beetschen, Zalta & Duprat 1965; and Love 1966) but have been widely reported in ultrastructural studies. They have been described as 'coalescence', 'redistribution', 'segregation' (Shoefl 1964; Jacob & Sirlin 1964; Stevens 1964, and Lane 1969) or as 'nucleolar cap

formation' (Reynolds, Montgomery & Hughes 1964). The process of nucleolar segregation in vertebrate cells is usually described as a peripheral condensation of the granular (cortex) component and a subsequent separation of granular, fibrillar and amorphous zones in the nucleolus (Shoefl 1964, Reynolds et al. 1964, Suter & Salomon 1966; Geuskens 1966; Geuskens and Bernhard 1966 and De Man & Noorduyn 1967).

Low concentrations (0.1 - 10µg/ml) of Actinomycin D have generally been sufficient to cause complete nucleolar segregation in most vertebrate somatic cells, the rearrangement of components taking place fairly rapidly (30 mins - 7 hrs.). In amphibian tissues a higher concentration or a longer duration of treatment are necessary; Burns (1968) showed that at concentrations below 10µg/ml nucleolar segregation in lung cells from Rana pipiens and Triturus viridescens may take as long as 3 days but at 100µg/ml complete emptying of the nucleolus occurs in about 3 hrs. Lane (1969) reports segregation of oocyte nucleoli in T.viridescens after 12 to 24 hrs. treatment with 50-100µg Actinomycin D/ml.

MATERIAL AND METHODS

The newts used for the chromosome studies were mature females of the crested newt Triturus cristatus cristatus. Details concerning their keep and the procedures for administration of Actinomycin D, and for chromosome isolation and study are described by Snow & Callan (1969).

For the observations made on fixed material the subspecies T.c.carnifex was used in preference to cristatus as the latter animals were in short supply. T.c.carnifex females were collected near Naples, Italy and supplied

by Dr P. Dohrn. They were kept in the same manner as cristatus females.

Oocytes selected for chromosome studies were in the size range 0.7 - 0.9mm diameter but for studies on nucleoli and on RNA metabolism oocytes of up to 1.1mm diameter were used.

RNA metabolism was studied by autoradiography of H^3-uridine. H^3-uridine, specific activity 760mC/mM was purchased from the Radiochemical Centre, Amersham. It was administered as a single subcutaneous injection of 200μC given in a ventro-lateral position about 1cm in front of the hind limbs. All preparations made were washed for 5 minutes in cold 5% Trichloracetic acid (TCA) to remove surplus precursors. Autoradiographs were prepared using Kodak dipping emulsion, NTB-2, for chromosome preparations or oocyte sections less than 3μ thick, and Kodak stripping film, AR-10 for sections thicker than 3μ. After suitable exposure time they were developed in Kodak D-19b and fixed in Kodak Metafix. Autoradiographs of chromosome preparations were examined wet and under phase-contrast optics (see Snow & Callan, 1969) but oocyte sections were stained through the film after fixation. NTB-2 coated preparations were stained for 10 mins. in 0.00375% Toluidine Blue in M/1000 phosphate buffer pH 6.0; AR 10 coated material was stained for 20 mins. in 0.15% methyl green in 0.1M Acetic acid/Sodium acetate buffer at pH 4.7. They were covered with $N^{o}0$ cover glasses mounted in Euparal.

Fixed material was prepared in several ways. The fixatives used were either Sanfelice's Fluid or 10% neutral formalin. The material was embedded either in wax or in methacrylate. Methacrylate was preferred for

yolky eggs. Sanfelice's fixative was avoided when material was to be prepared for autoradiography since the chromium interferes with the extraction of unincorporated precursors by cold TCA and also can result in latent image fading in autoradiographs (Callan, personal communication). Wax embedded material was cut on a Leitz rotary microtome to give a section thickness of 8 μ to 12μ; methacrylate embedded material was cut with a glass knife on a Porter-Blum Ultramicrotome to give sections 1 or 2 μ thick.

Haidenhains Iron Haematoxylin was used as a general nuclear stain and preparations were mounted in Euparal. RNA distribution was studied using the Azure B bromide technique of Flax & Himes (1952) or the gallocyanin chrome alum technique described by Swift (1955). These preparations were mounted in DPX synthetic mounting medium and Euparal respectively. Material examined by phase-contrast optics was not stained but was mounted in Zeiss Phase-contrast mounting medium, refractive index 1.525.

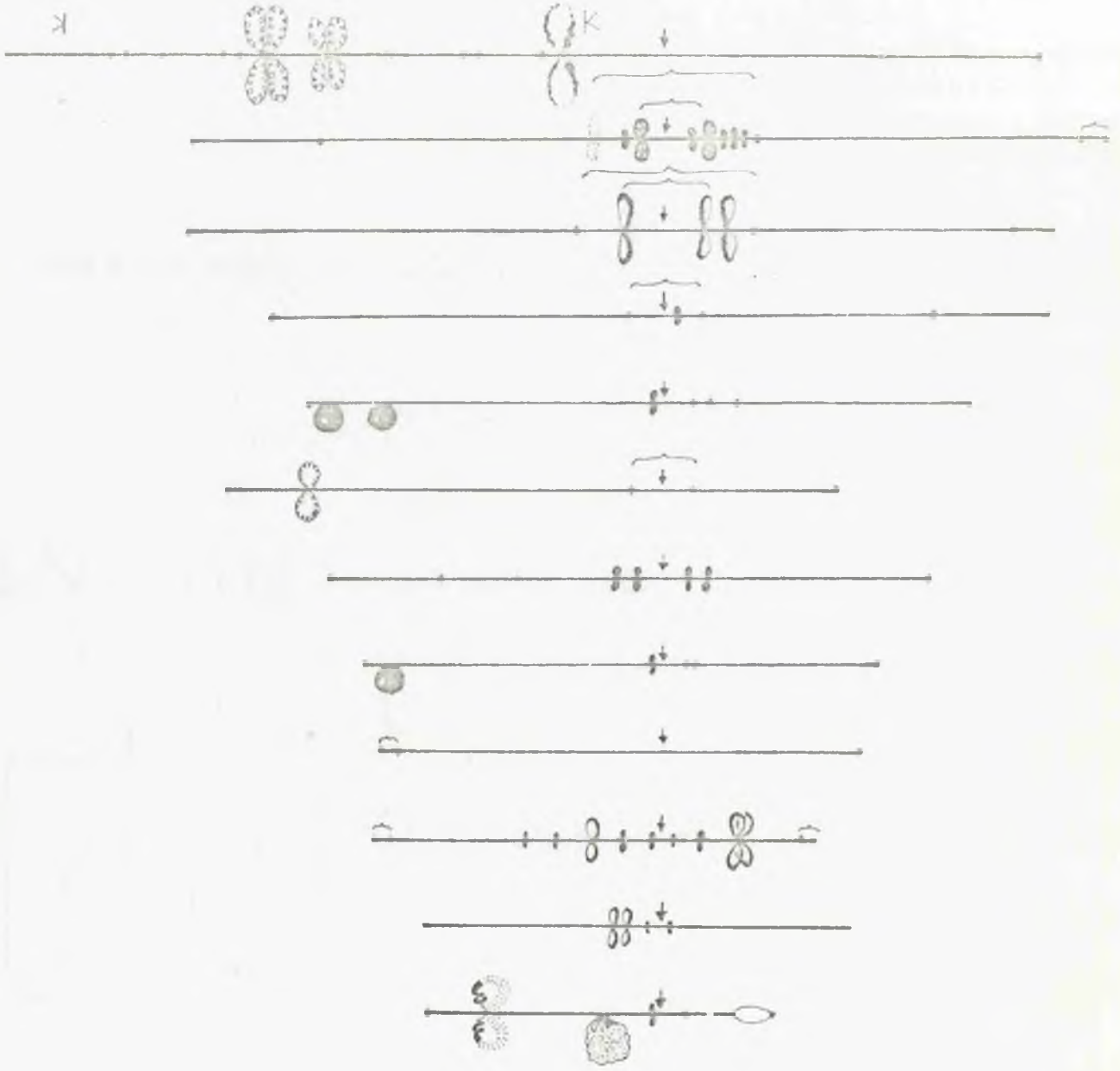
Oocyte material for electron microscopy was fixed for 20 mins. in 10% glutaraldehyde in phosphate buffer pH 6.5, and post-fixed for 60 to 90 mins. in 1% osmium tetroxide made up in Veronal/0.1N HCl buffer pH 7.2 - 7.5. Subsequently dehydrated through a graded series of acetones, with three changes of water-free absolute acetone, the material was embedded in Vestopal-W polyester. Grey or silver section ($< 90 m\mu$) were cut with a glass knife on a Huxley type ultramicrotome and collected on copper grids.

They were stained for 5 minutes in 2% Uranyl acetate and 2 minutes in 0.3% lead citrate, and examined in a Siemens Elmskop I Electron microscope.

Freshly isolated preparations of lampbrush chromosomes were examined by means of a Zeiss inverted Plankton microscope fitted with phase contrast optics and photographed using a Zeiss Ukatron flash unit. Fixed material was examined using a Zeiss GFL microscope fitted with the appropriate optics. Photographs were made using a Zeiss Photomicroscope with automatic exposure.

Chorionic gonadotrophin (CG) pregnyl was obtained in powder form from Organon Laboratories Ltd. London. The method of application is described below.

X-irradiation was carried out in the Radiotherapy Department of Dundee Royal Infirmary using a 60kv 5ma source delivering 200 roentgen/ minute.



Text figure 1. Working map of the lampbrush chromosomes of T.c.cristatus (taken from Callan & Lloyd, 1960).

RESULTS

Chromosome Studies

Text Figure 1 is a working map of the lampbrush chromosomes of T.c.cristatus (reproduced from Callan & Lloyd 1960) showing the relative length of the twelve members of the chromosome complement, the position of their centromeres and of the major marker loops. The longer chromosome arm is drawn projecting to the left thus defining the left-hand end of the chromosome. The marker loops selected for study, representative of the different types of marker loop, were as follows.

1) The Giant normal loops on the left hand arm of Chromosome I. Figure 2 shows an example of one of these loops.

2) The lumpy loops in the centromere region of chromosome II. Figure 5 illustrates this group of loops.

3) The Giant fusing loops to the left of the centromere of chromosome XII. They are illustrated in figure 8.

4) The spheres on the left hand end of chromosome V. There are two spheres in this position and they are shown in figure 12.

5) The giant granular loops on the left hand arm of chromosome XII. These loops are dealt with in detail by Snow & Callan (1969). In this section they will be considered only with regard to gonadotrophin treatment.

As the immediate result of a 4 hr. treatment with 100µg Actinomycin D/ml *in vivo* the marker loops show some small changes. The giant normal

loops, lumpy and fusing loops all show a contraction in length or volume. The spheres remain unaltered by Actinomycin D. After removal from Actinomycin D these marker loops undergo a variety of different changes.

1) Giant normal loops

The reduction in length of these loops during antibiotic treatment is some 30-50% (Fig. 5) and the reduction continues during the first day after treatment. 24 hrs. after removal from Actinomycin D it is not possible to identify these loops. During this time some 20μ of the normal lateral loops have re-appeared and it would be impossible to identify a small "giant loop" from the surrounding lateral loops. The presence of the giant normal loops can nevertheless be inferred from the fact that all chromomeres in the region display pairs of lateral loops at this time. Two days after treatment the giant normal loops have recovered to a considerable degree but are still shorter than in untreated oocytes (Fig 4.). After 4 days of recovery they are indistinguishable from the giant normal loops of controls.

At no time do the giant normal loops show any irregularity in their matrix. In autoradiographs they have not shown the partial labelling seen in occasional normal lateral loops (Snow & Callan 1969).

2) Lumpy loops

These loops generally undergo no great change as a result of Actinomycin D treatment (Fig. 6). Any contraction in volume during

antibiotic treatment is corrected during the first day after treatment. Thereafter there was usually no change. However, in one animal, oocytes examined 14 days after treatment possessed enlarged lumpy loops. This phenomena is shown in Fig. 7. The reason for this departure from the normal course of recovery is unknown. These loops retain some if not all, their RNA during Actinomycin D treatment, probably because, in common with the other marker loops, the matrix is dense enough to prevent immediate dispersal of RNA.

3) Giant fusing loops

The fusing loops behave in precisely the same manner as the lumpy loops, but generally show a greater reduction in size during Actinomycin D treatment. Figure 9 shows an example of a small fusing loop following Actinomycin D treatment. Two days after treatment most of these fusing loops appear to be completely normal although the occasional small fusing loop can still be found. Three days after treatment all these loops have returned to the conditions found in the untreated oocyte and, with one exception, showed no further change. In the same animal that produced enlarged lumpy loops 14 days after treatment the giant fusing loops were also enormously swollen (Fig.10).

Autoradiographs of incorporated H^3-uridine show that the RNA synthesized immediately prior to Actinomycin D treatment is retained. Figure 11 shows an autoradiograph of a preparation made immediately after Actinomycin D treatment from an animal given 12 hrs. availability of

H^3-uridine before treatment.

4) Spheres

There is no apparent change in these structures during or after Actinomycin D treatment. Figure 13 shows spheres immediately after antibiotic treatment, and Figure 14 spheres one day after treatment.

Gonadotrophin treatment

Four animals were used to investigate the effect of differences in the rate of RNA synthesis, upon the time course of Actinomycin D treatment and the subsequent recovery therefrom. In this study particular attention was once again paid to the giant granular loops of chromosome XII.

Chorionic gonadotrophin was used to accelerate the rate of RNA synthesis. Each experimental animal was given a subcutaneous injection of 200 i.u. of hormone in 0.1 ml sterile water and 5 days later a second similar injection was made. 24 hrs. after the second injection the ovaries were exposed to 1, 2 or 4 hrs. of Actinomycin D treatment.

The acceleration in RNA synthesis does not result in an increase in the rate of matrix stripping from the lateral loops of the lampbrush chromosomes. Nor is there a change in the rate of the recovery process in any of the loops studied. The only observable difference between hormone treated animals and those given Actinomycin D without gonadotrophin is seen in the giant granular loop. In hormone treated animals there is an increased tendency for the granular portion of this loop to collapse or disintegrate

during recovery from Actinomycin D treatment. The long filament produced during recovery from Actinomycin D treatment (see Snow & Callan) is the same length and it does not grow or acquire matrix any more rapidly than in the absence of gonadotrophin stimulation. The collapse of the granular portion of these loops makes measurement of the post-Actinomycin D filament very difficult. One animal yielded 6 well displayed giant granular loops in preparations made one day after treatment. Measurements of the filament made from these drawings range from 14 to 22μ with a mean length of 16.3μ. This value compares very well with the extension rate given in Snow & Callan 1969. Preparations made 2 days after treatment show the filament to be in the usual +2 day condition.

It is not known if, in the long term, the transformation of the long filament into a giant granular loop is accelerated by gonadotrophin treatment. Only one animal survived beyond +17 days and when examined 14 days after Actinomycin D treatment was in an extremely poor condition and it was decided that any chromosome preparations made would not yield trustworthy information.

The effects of X-irradiation

A 1000 roentgen dose of X-irradiation was selected as the best compromise between a lethal dose and one too low to cause a significant number of lateral loop breaks. Of the three experimental animals one died 6 days after irradiation, one was sacrificed after 7 days and the third after 12 days of recovery.

Lampbrush preparations were made before Actinomycin D treatment from 2 animals. The 16 preparations from animal S14 showed an average of 48.4 $\pm$ 5.25 breaks per preparation, and the 10 preparations from animal S15 showed 49.0 $\pm$ 4.49 breaks per preparation. Since these analyses were based upon the presence of visible broken loop ends it is probable that some breaks, in very small loops were missed. It must also be borne in mind that, despite the considerable care taken in making these lampbrush preparations, physical damage during isolation may be responsible for some of the recorded breaks. Since it is possible to make normal lampbrush preparations with less than 10 breaks visible it is extremely probable that most of the recorded breaks are X-ray induced and present in situ.

4 hrs. of Actinomycin D treatment resulted in complete disappearance of normal lateral loops. One day after irradiation and Actinomycin D treatment over 50% of the small and medium sized oocytes were moribund but lampbrush preparations from the healthy oocytes showed that recovery was following the usual course.

During the 12 days after X-irradiation the numbers of healthy oocytes falls rapidly and after 12 days the entire ovary of the one surviving animal was moribund. Lampbrush preparations made after 4 and 7 days of recovery from Actinomycin D treatment still show that the healthy oocytes follow a normal course of recovery. There is no evidence during this recovery process for any lateral loop breaks that may have been caused by X-irradiation and persisted during the recovery process.

Nucleolar studies - light microscopy

Throughout the studies on isolated nuclear contents the nucleoli showed no morphological change that could be associated specifically with Actinomycin D treatment. Treated oocyte nucleoli did show an increased tendency to vacuolate, or to separate into core and cortex upon isolation but many different factors can cause such behaviour including prolonged immersion of oocytes in Ringer solution.

Autoradiographs of isolated nucleoli from oocytes given 12 hrs. availability of H^3-uridine indicate considerable incorporation of precursors. Figure 15 shows an example of such nucleoli. During Actinomycin D treatment the nucleoli lose the RNA synthesized immediately before treatment, (see Figure 16) but rapidly recover the ability to incorporate H^3-uridine after treatment such that after one day of recovery they are once again heavily labelled (Fig. 17).

Sections through untreated oocytes, stained with iron haematoxylin, Azure B bromide or Gallocyanin chrome alum show nucleoli in which no marked zonation can be observed (Fig. 18 - 20). Nor is zonation apparent under phase contrast optics (Fig. 21). After Actinomycin D treatment oocytes above 0.7mm diameter contain medium and large-sized nucleoli most of which, cut perpendicular to the nuclear membrane, exhibit a characteristic zonation when stained with haematoxylin, or when viewed under phase contrast optics (Fig. 22 - 26). The nucleolus appears to have segregated into two zones which stain with different intensities. The darker, or more

contrasted, zone, usually crescent shaped, is always directed towards the centre of the nucleus. There occasionally appears to be a clear region separating these two zones which may represent a type of vacuolation. Figure 24 illustrates this point. In Azure B bromide stained sections it is extremely unusual to find any evidence for nucleolar zonation. Where it has been seen it does not conform to the description above. Zonation in these cases is irregular and ill-defined. Figures 27 and 28 show Azure B stained preparations exhibiting zonation. Gallocyanin staining on no occasion revealed zonation of nucleoli (Fig. 29 and 30). This is an interesting finding since Macgregor (personal communication) has found Gallocyanin to be a very sensitive stain for demonstrating RNA distribution in nucleoli. For comparison Fig. 31 shows a section through an untreated oocyte, of comparable size, from Bufo bufo stained with Gallocyanin. The differentiation of core and cortex is very well marked.

After ribonuclease treatment nucleoli do not stain at all with Azure B or Gallocyanin but the zonation of the nucleolus is still apparent in haematoxylin stained sections. (Fig. 32).

Although I have never observed a marked zonation in nucleoli of untreated oocytes, Macgregor (1967) in figures 2 - 4 shows such a zonation in sections of methacrylate embedded material prepared for autoradiography and stained with methylene blue.

In oocytes sampled 1 day after Actinomycin D treatment the nucleolar

zonation is considerably reduced (Fig. 33 - 36), and cannot be observed 2 days after treatment, when the nucleoli are indistinguishable from those in untreated oocytes.

Autoradiographs of sections through oocytes that have incorporated H^3-uridine indicate that during Actinomycin D treatment almost all recently synthesized RNA leaves the nucleolus. Figure 37 and 38 show a preparation made immediately before Actinomycin D treatment and after a 3 day availability of H^3-uridine. Figures 39 and 40 show comparable preparations after the 4 hr. Actinomycin D treatment.

After Actinomycin D treatment dehydration of fixed material with acetone prior to methacrylate embedding causes considerable contraction of nuclear contents. Figures 38 and 39 show sections through comparable oocytes before and after Actinomycin D treatment. In some extreme cases this shrinkage is so great that the nuclear membrane only maintains contact with the cytoplasm in a few places.

Electron microscopy

The peripheral nucleoli of Triturus cristatus do not show a very well defined ultrastructural zonation into core and cortex. Untreated nucleoli are composed of granules, 150-200A° diameter among which scattered groups of fibrils, about 100A° thick, can be located. Figures 41 - 43 show these components, both of which are embedded in an amorphous matrix. In untreated mucleoli in which a zonation is apparent the core is usually eccentrically situated nearest to the nuclear membrane. It shows a higher

concentration of granules and fibrils than the cortex (Fig. 41 and 43).

Some 20-30μ inside the nucleus there is a region containing large numbers of spheroidal bodies, less than 1μ in diameter. They occur in all oocytes in the size range examined (0.6 - 1.1mm diameter) and do not represent sections through parts of 'ring nucleoli' (see Lane 1967). For reasons that will become apparent later these bodies will be termed micronucleoli. They are composed of granules 25-50A° in diameter and fibrils of similar thickness. The granules show a tendency to form aggregates some 200A° across composed of 20-50 smaller sub-units. Figures 44, 45 and 46 show examples of these micronucleoli. It can be seen that there is some evidence for a continuity between the nuclear sap and these bodies. Very occasionally a micronucleolus has been found in a peripheral position, immediately adjacent to the nuclear membrane, but never have they been observed in the intervening region.

After the standard 4 hr. Actinomycin D treatment the peripheral nucleoli are reduced in size and are found to consist chiefly of fibrils 50A° thick embedded in a finely granular matrix (Figures 47 - 49), the 200A° diameter granules have been lost. The nucleolus has become difficult to stain and in order to obtain sufficient contrast within the section, the staining times were extended to 10 mins. in uranyl acetate and 5 minutes in lead citrate. In one animal, oocyte samples were fixed after 2 hrs. of Actinomycin D treatment. The nucleoli of these oocytes show exactly the same features as found after a 4 hr. antibiotic treatment. The

Actinomycin induced changes were already completed.

1 μ thick sections of this treated material, stained with methylene blue and borax, do not show nucleolar zonation when examined in the light microscope.

The micronucleoli undergo a considerable change during Actinomycin D treatment, exhibiting all the characteristics of classical nucleolar segregation. There has been a concentration of the granular aggregates to one side, or around the periphery, of the micronucleolus. These aggregates form the electron dense regions seen in Figures 50 and 51 and may now be some 300-400A° across. In the less dense region, granules 30-50A° diameter are embedded in a fibrillar network. The fibrils are 30-50A° thick (Fig. 52). In many cases the nucleoprotein network of the nuclear sap appears to be continuous with and to be streaming either into or away from the micronucleolus. This phenomenon is readily visible in Figure 51.

Also present in the nucleus of Actinomycin D treated oocytes there are many rod-shaped bundles of what appear to be fibrils, similar to those found by Lane (1969) and named fibrillar bodies. In this study these bodies reached a length of 3-4μ and a diameter about 0.4μ. They were generally too thin to be observed in the light microscope. Figures 53, 54 and 55 show examples of these bodies. The transverse sections shown in Figs. 55 and 56 indicate that they are composed of lamellae and not fibrils. These lamellae measure 160A° wide and 40A° thick; the

centre to centre spacing is 80-100 A°. It can be seen in Figures 53 and 54 that these lamellar bodies also appear to be continuous with the nucleoprotein network of the nuclear sap, and it is possible that they represent a form of crystallization or condensation of nucleoprotein.

One day after Actinomycin D treatment the granules of the peripheral nucleoli have reappeared but are now only 100-150A° diameter and although the nucleoli show considerable vacuolation, in many instances they appear to be recovering from Actinomycin D poisoning. This can be clearly seen in Figs. 57 and 58. The micronucleoli at this stage are seen to have lost a great deal of material. The granular aggregates have been lost and small particles about 30A° in diameter embedded in a network of fibrils 30-50A° thick is all that remains (Figs. 59 and 60). The lamellar bodies have vanished, presumably dispersed.

Two days after treatment the peripheral nucleoli and micronucleoli are essentially indistinguishable from the untreated condition. At this stage, there is no evidence in electron micrographs to indicate that the oocyte had been treated with Actinomycin D. (Figs. 61 and 62)

DISCUSSION

Lampbrush Chromosomes

Compared with the behaviour of normal lateral loops and the giant granular loops during and after Actinomycin D treatment the changes induced in the other marker loops is relatively unspectacular. The inertness of the spheres of Chromosome V is the easiest to explain. It has been demonstrated that these structures are Feulgen negative, do not incorporate H^3-uridine and seem to be composed entirely of protein (Callan & Lloyd 1960, and Callan 1963), although the nature and origin of this protein is unknown. The behaviour of the lumpy loops and the fusing loops is most probably a reflection of the nature of their matrix. Macgregor (personal communication) found that these loops respond only slowly to digestion by pepsin, trypsin and RNase and inferred that this was attributable to the high density of their matrix. The action of Actinomycin D is solely to prevent the continuation of DNA-dependent RNA synthesis. The absence of any lateral loop matrix stripping in chromosomes isolated directly into an Actinomycin D solution (see Snow & Callan 1969) indicates that no physicochemical removal of matrix occurs but that matrix loss is dependent upon the continuation of a physiological process in the absence of matrix replacement. Under these conditions loss of lumpy loop and fusing loop matrix would be expected to be slow. Bearing in mind that RNA synthesis is resumed very rapidly after removal of Actinomycin D the point at which maximum reduction in matrix occurs,

a few hours after treatment, may be scarcely noticeable. The density and morphology of the matrix would similarly prevent the extension of loop axis in the manner already described for the giant granular loops.

The giant normal loops of chromosome I form an intermediate condition between normal loops and lumpy/fusing loops. Whereas their matrix is sufficiently bulky for stripping not to be completed during the 4 hrs. incubation there is nevertheless a considerable reduction in length as a result of Actinomycin D treatment.

In the observations reported on the giant granular loops the origin of the post-Actinomycin D filament was discussed. The evidence presented by Snow & Callan (1969) favours the chromomere as the source of this filament but does not definitely rule out the possibility that it arises from the dense end of the old granular loop. At first sight the results of the gonadotrophin/Actinomycin D experiments reawaken interest in the dense end of the giant granular loop. Under the conditions of the experiment it might have been expected that gonadotrophin would accelerate the rate of extension of the filament were it indeed of chromomeric origin and result in an increase in the maximum length of the filament. This manifestly is not the case but bearing in mind that none of the recovery process is accelerated, the possibility that all the relevant metabolic functions are already proceeding at their maximum rate cannot be excluded. For this reason the observations made with dilute saline (Snow & Callan 1969) are considered much more significant.

It was to investigate the growth of lateral loops that the effects of X-irradiation were studied. Despite the especial care taken when making lampbrush preparations after X-irradiation it cannot be claimed with complete certainty that the loop breaks recorded after treatment were X-ray induced as assessment of the damage due to isolation may be subject to optimism. In order to identify the original lateral loop breaks after Actinomycin D treatment it was necessary to allow 6 or 7 days recovery. After 3-4 days the normal lateral loops will have recovered to their original length and it is only after a further few days recovery that an excessive length of lateral loop with a single, thin insertion could be positively identified as a continuously extending lateral loop rather than a lateral loop broken very close to its thicker insertion.

The absence of such loops in the preparations analysed at a suitable time needs consideration. There are several possible explanations. First, the oocytes surviving to the time of sampling would probably be those least damaged by X-rays and may not have sustained any lateral loop breaks during irradiation. Secondly, the in situ breaks were rapidly repaired during the Actinomycin D treatment or at the chromomere level. Thirdly, it is possible that the lateral loop axis that emerges after Actinomycin D treatment is not the DNA axis that was previously extended from the chromomere.

Of these postulates I think the first can be discounted as the initial

sampling after irradiation failed to reveal any undamaged oocytes. The second postulate must be regarded as doubtful as the movement of loop axis during Actinomycin D treatment would surely prevent repair of the loop axis. However, the possibility of repair within the chromomere cannot be ruled out. The third possibility is, I feel, the most likely and, as discussed by Snow & Callan (1969), provides the only plausible explanation for the occasional occurrence of loops only partially labelled with H^3-uridine in autoradiographs made during the first few days of recovery.

Nucleoli

There is no reason to believe that the peripheral nucleoli of Amphibian oocyte nuclei are functionally very different from the nucleoli of other cell types but there is evidence to suggest that they are involved in ribosomal RNA production to a lesser extent than, for example, mammalian nucleoli.

Girard, Penman & Darnell (1964); Girard, Penman, Latham & Darnell (1965) and Perry (1965) demonstrated that the 18s ribosomal RNA fraction labels earlier than the 28s fraction in Hela cells and L-cells and discuss a scheme whereby the 18s RNA molecule is released at the time the 45s precursor is converted to the 35s molecule. The 28s ribosomal RNA is then formed directly from the 35s molecule. Since the 28s ribosomal unit and the 45s precursor are found in the nucleoli of mammalian cells (Busch, Desjardins, Grogan, Higashi, Jacob, Muramatsu, Ro & Steele, 1966)

it would appear that the production of the ribosomal RNA is completed within the nucleolus in some cases.

While Gall (1966) agrees in principle that the same process occurs in oocyte nuclei from Triturus viridescens he failed to find significant quantities of newly synthesized 28s and 18s ribosomal RNA's in the nuclear extracts. Gall found that recently synthesized nuclear RNA showed sedimentation values of 40s and 30s and equates these molecules with the 45s and 35s precursors of mammalian cells. Taking into account the distribution of RNA in the nucleus (Izawa, Allfrey & Mirsky 1963 (b) and Edstrom & Gall 1963) Gall argues that conversion of the 40s precursor to the 30s precursor takes place in the nucleolus with the release of the 18s ribosomal fraction which migrates immediately to the cytoplasm. The 30s precursor probably leaves the nucleolus and completes its metamorphosis to the 28s ribosomal fraction in the nuclear sap, almost concomitantly with the migration of the latter to the cytoplasm.

Perry (1962) showed that low concentrations of Actinomycin D selectively inhibited the synthesis of ribosomal (nucleolar) RNA in L-cells. As a result of this inhibition there followed a deficiency of labelled 45s and 35s RNA precursors in cell extracts. His conclusion, that inhibition of nucleolar RNA synthesis does not interfere with the conversion of 45s precursor to the 35s and the coincidental production of the 18s ribosomal fraction has been substantiated by Girard et al.

(1964), Scherrer & Darnell (1962) and Scherrer, Latham & Darnell (1963). The conversion of the 35s precursor to the 28s ribosomal molecule, however, is either inhibited or abnormal in the presence of Actinomycin D (Penman, Smith, Holtzman & Greenberg, 1966).

The process of maturation of the ribosomal fractions from the 45s RNA precursor usually takes 60-90 mins. (Penman et al. 1966, Perry 1962 & 1966). Since the above described observations involve a 4 hr. incubation with Actinomycin D the changes concerned with the metamorphosis of the 40s ribosomal precursor will most probably have been completed during the antibiotic treatment. The loss of H^3-uridine labelling from nucleoli as a result of treatment is strong evidence for Gall's hypothesis that the 30s precursor leaves the nucleolus. If the conversion of the 30s precursor to the 28s ribosomal molecule were to take place in the nucleolus then the prevention of this step by Actinomycin D might be expected to result in retention of H^3-uridine labelling in the nucleolus. Shoefl (1964) found that incorporated H^3-uridine is not lost from nucleoli of baboon kidney cells during a 7 hr. incubation in Actinomycin D concentrations that totally inhibit RNA synthesis. This does not occur in T.cristatus oocyte nucleoli. Nevertheless the possibility of abnormal breakdown of this RNA species cannot be ruled out. Harris (1963) and Schwarz & Garofalo (1967) present evidence for the intranuclear breakdown of RNA in the presence of Actinomycin D.

The maturation of the 40s precursor and migration of the 30s precursor

from the nucleolus could account for the large loss of the granular component seen in electron micrographs of Actinomycin D treated nucleoli. Unfortunately there is at present no direct evidence to link specific nucleolar structures with specific RNA molecules.

It is at first sight puzzling that the typical nucleolar segregation associated with Actinomycin D poisoning has not been observed in the peripheral nucleoli of T.cristatus. Bearing in mind that segregation has been reported in T.viridescens for oocyte nucleoli (Lane 1969) and for lung cell nucleoli (Burns 1968) it is possible that segregation has been completed or has not yet occurred in T.cristatus nucleoli. There are several reasons why I think neither of these explanations is true but that morphological segregation of the type described by Shoefl (1964) genuinely does not occur in the peripheral nucleoli of these oocytes. First, in the oocytes examined after a 2 hr. Actinomycin D treatment there is no evidence for a re-arrangement of components prior to the loss of granular material. It is extremely doubtful that the process of segregation would have been completed in less than 2 hrs. Burns (1968) reports that lung cell nucleoli of T.viridescens require a minimum of 3 hrs. to complete their segregation in incubated in vitro with 100µg Actinomycin D/ml of culture medium and in vivo systems are generally less susceptible to Actinomycin D poisoning (Flickinger, 1963; and Harel, Harel Boer, Imbenotte & Carpari, 1964). Secondly, during the studies on isolated lampbrush chromosomes some 10% of the oocytes examined after Actinomycin D treatment

showed a variable degree of stripping. These cells were regarded as being poorly supplied with blood, in a localized region of the ovary where the ligation of the blood supply was inadequate. In the 100 or more oocytes, from 6 different animals, examined in the ultrastructural studies some such oocytes, representing mild Actinomycin D treatment, may have been encountered. If so, these oocytes do not show segregation of the peripheral nucleoli.

Almost certainly, therefore, typical nucleolar segregation does not occur during Actinomycin D treatment. The possibility of it occurring soon after treatment can be ruled out on the grounds that the granular component has already been lost from the nucleolus and rearrangement of components has therefore become impossible.

At this point it is appropriate to ask what has become of the granular component of the nucleolus? The 200A^{o} diameter granules are composed of RNA and protein (Marinozzi, 1964) the bulk of which is regarded as protein. The absence of any evidence for the granular fraction of the nucleolus, anywhere in the nucleus, after Actinomycin D treatment points to two possible fates. Either the granules have broken down or they have migrated to the cytoplasm. The second possibility is unlikely; Girard et al. (1964) and Schwarz & Garofalo (1967) have demonstrated that in mammalian cells the passage of RNA from nucleus to cytoplasm is inhibited by Actinomycin D.

It seems probable therefore that the granular nucleolar component is broken down, but is there any evidence for breakdown products? Autoradiography of nuclear sections after Actinomycin D treatment clearly cannot separate nucleolar RNA and protein from the chromosomal and nuclear sap material. Any loss of H^3-uridine labelling from the nuclear sap following Actinomycin D treatment can readily be explained in terms of degradation of chromosomal RNA. Cytologically it is not possible to identify or locate nucleolar RNA breakdown.

It has been suggested by Lane (1969) that the lamellar bodies found in the nucleus after Actinomycin D treatment may represent accumulation of ribosomal protein since they apparently contain neither DNA or RNA, but there is not a noticeable spatial relationship between lamellar bodies and nucleoli, In her study Lane describes the lamellar bodies as fibrillar structures and illustrates a transverse section of such a body. It is possible that the differences between the fibrillar bodies of that study and the lamellar bodies described above are attributable to the different duration of antibiotic treatment of the two studies, but I think that Lane's figures 6 and 7 are more likely to be sections through small, severely damaged, nucleoli.

The appearance of lamellar bodies must be regarded as an indirect result of Actinomycin D poisoning. Mann (1894) reported intranuclear rods in dog nerve cells stained with methylene blue. More recently ultrastructural studies on mammalian nerve cells have similarly found intranuclear rods

in a small percentage of cells, (Siegesmund, Dutta & Fox 1964; Popoff & Stewart 1968; and Hirano & Zimmerman 1967). Such structures have also been found in normal human thymus cells (Henry & Petts 1969) and in kidney cells of monkey infected with SV40 virus (Granboulan, Tournier, Wicker & Bernhard 1963). All the above mentioned ultrastructural reports show the intranuclear rods to be indistinguishable from the lamellar bodies I have described above. A very similar, lattice like structure has been found in neurons of rats and mice (Chandler 1966 and Chandler & Willis 1966). It is noteworthy that the cells in which these rods have been found are also cells rich in and normally actively synthesizing RNA.

Bearing in mind that only a small percentage of the cells possessed the intranuclear rods the question that immediately arises is whether these cells are in fact normal and healthy. If they are, it is unlikely that the lamellar bodies represent break down products from a defunct nuclear metabolic process. It is more probable that they represent an accumulation of a particular protein, such as RNA polymerase or a structural protein, that was required to meet the demands of periods of intense synthetic activity, and produced in excess. Such conditions would arise by the sudden cessation of e.g. RNA synthesis and the small delay that would occur before the influx of enzymes and structural proteins into the nucleus stopped. Such bursts of activity are an obvious feature of nerve cells, and thymus cells also appear to undergo cyclical changes in their activity (see Everett & Tyler 1967).

Further insight into the nature of the lamellar bodies might be obtained by biochemical analysis of the cells in which they occur, or by investigating whether or not they are formed in the presence of a protein synthesis inhibitor such as puromycin or cyclohexamide. Such analyses could help decide if these bodies are breakdown products and may provide further insight into the question of their origin.

Turning once again to Gall's scheme for ribosome biogenesis, the site at which the 30s precursor is converted to a 28s ribosomal RNA molecule must be sought. It is possible that the precursors are randomly distributed throughout the nuclear sap but I think this unlikely. Haphazard organization is not a characteristic of biological systems. Nevertheless, if the 30s precursor carries with it all the requisite material for production of the ribosomal particle with its 28s RNA molecule, then an organized site for the conversion would be unnecessary. The primary action of Actinomycin D is the inhibition of DNA dependent RNA synthesis (Reich & Goldberg 1964; and Reich, Cerami & Ward 1966), and protein synthesis is affected as a second order phenomenon (Honig & Rabinowitz 1964; Spector & Kinoshita 1965, and Bannerjee, Flamm & Counts 1966) Either of these effects points to the abnormal conversion of the 30s precursor in the presence of Actinomycin D, being due to the lack of an essential component, either an RNA molecule or a protein (a structural protein or an enzyme). The incorporation or involvement of this extra component would be greatly facilitated by the existence of specific

conversion sites.

In this respect the presence and behaviour of the micronucleoli is of interest. Although any site for the final maturation of ribosomal particles might be expected to be located very close to the nuclear membrane it is perhaps significant that the micronucleoli undergo a segregation that is precisely similar to that exhibited by nucleoli of cell types in which final production of the ribosomal subunits is regarded as a nucleolar function. Could the process of nucleolar segregation be the morphological manifestation of the abnormal metamorphosis of the smaller ribosomal precursor?

The nucleolar zonation seen after Actinomycin D treatment in preparations made for light microscopy is in no way similar to that seen in the ultrastructural studies. It was at first thought to be due to a redistribution of RNA but the failure to demonstrate this with Azure B, Gallocyanin, or by autoradiography must rule out this explanation. Bearing in mind that contraction of nuclear contents is a recognized fixation artefact following Actinomycin D treatment (Simard 1966, and Burns 1968) it is most likely that this is the explanation for the light microscope observations. Nevertheless, it is noteworthy that the nucleolus splits into two zones under these conditions and stimulates speculation about the protein composition of the two regions.

SUMMARY

The *in vivo* action of Actinomycin D upon the giant normal loops, the lumpy loops, the fusing loops and the spheres of the lampbrush chromosomes in *T.c.cristatus* is described. Small contractions in size have been observed in all but the spheres as a result of a 4 hr. antibiotic treatment. Recovery from Actinomycin D poisoning is rapid in these loops, being completed within 2 days.

Newly synthesized RNA is not lost from these marker loops during Actinomycin D treatment.

Chorionic gonadotrophin does not accelerate nor accentuate the normal *in vivo* effects of Actinomycin D upon these lamporush chromosomes nor does it accelerate the recovery process.

X-irradiation causes *in situ* breaks in the lateral loops of lampbrush chromosomes, which become evident only upon mechanical separation of the ends of the fractured DNA loop axes. They are not revealed by Actinomycin D treatment following irradiation. *In situ* X-ray induced breaks are either rapidly repaired, or are concealed within the chromomere after Actinomycin D treatment.

The literature pertaining to a study of nucleoli and the effects of Actinomycin D upon this organelle is reviewed. After Actinomycin D treatment a marked zonation of the nucleolus is evident in oocyte sections prepared for light microscopy. The zonation does not represent a change in RNA distribtuion within the nucleolus but probably reflects a segregation

of nucleolar protein. As such a zonation is not apparent in material prepared for ultrastructural studies it is regarded as a fixation artefact.

Classical ultrastructural nucleolar segregation has not been observed in peripheral nucleoli and is presumed not to occur. There is however, a loss of the granular component of the peripheral nucleoli as the result of a short incubation in high concentrations of Actinomycin D. The granular component gradually reappears during the first 2 days after antibiotic treatment.

The presence of numerous small spherical bodies (less than 1.0μ in diameter) within the nucleus has been observed. They have been termed micronucleoli. Under the influence of Actinomycin D the micronucleoli undergo a segregation of granular and fibrillar components exactly similar to those previously reported in nucleoli of other cell types. They return to their normal condition within 2 days of treatment.

The observation upon nucleoli and micronucleoli are discussed in the light of current theories concerning nucleolar function and ribosome production. It is postulated that the micronucleoli represent specific nuclear sites for the final conversion of the smaller ribosomal precursor molecule.

Also as a result of Actinomycin D treatment large crystalline structures, termed lamellar bodies, appear in the nucleus. They seem to represent a condensation of nucleoprotein, the precise nature of which is unknown. They disappear during the first day after Actinomycin D treatment.

Their production is discussed in the light of other reports of similar bodies in other cell types.

P A R T I I

INTRODUCTION

The time course of male meiotic prophase in Triturus vulgaris has been determined by Callan & Taylor (1968). They showed that, at 16°C, following a premeiotic DNA synthesis lasting 9-10 days, leptotene is completed in 5 days, zygotene in 8, pachytene in 4-5 and diplotene in less than 2 days. The total duration of meiotic prophase is therefore 20-21 days in this animal. Wimber & Prensky (1963) also made a comparable autoradiographic study of meiosis in T.viridescens. In their experiments the newts were kept at 20-22°C and 12 days elapsed before cells labelled at the end of premeiotic 'S' phase reached first metaphase. The difference in prophase duration in these two experiments is probably attributable to the different temperatures used in the two studies.

During the course of their investigation Callan & Taylor found that a small number of interphase nuclei seemed to show an abnormally high level of H^3-thymidine labelling. Subsequent investigations (Callan & Swallow, unpublished) carried out on testis sections showed that the more heavily labelled cells were in the spermatogonial interphases and the lighter labelled cells were in spermatocyte (pre-meiotic) interphase. Analysis of cyst numbers in the testis revealed that the level of labelling reflected the much longer duration of pre-meiotic 'S' phase as compared with the preceding inter-mitotic 'S' phases. Callan & Taylor concluded in their final paragraph that "It seems (to us) unlikely that an exceptionally slow rate of DNA synthesis results from limited availability of precursor nucleotides or polymerizing enzymes in spermatocytes. An alternative

explanation is that a major reorganization of chromosomes takes place during the interphases prior to meiotic prophase whereby either the size of the units of replication is changed or the rate at which units become available for the initiation of replication is changed. Techniques are now available which may allow investigation of these possible changes at the molecular level. The hypothetical repackaging could be part of an operation to ensure that recombination between homologous genetic material be restricted to 'master' nucleotide sequences as proposed by Callan (1967)."

To their hypothesis another can be added, also based upon the concept of 'master' and 'slave' nucleotide sequences. Since any correction of slave sequences must occur after genetic recombination and be completed before production of the mature gamete it might be expected that in closely related animals with large differences in gametic DNA content ('c' value) such a process would be reflected in differences in the time course of gametogenesis. In this respect the lampbrush phase of oogenesis is of particular interest as it is possible that the lampbrush phenomenon is the morphological manifestation of such a correction process (see Snow & Callan 1969).

The investigation described below set out to determine whether such differences could be found in the pre-meiotic 'S' phase or during the stages of meiotic prophase in selected male Amphibia.

Amphibians were chosen for this study because the class exhibits a very great range of 'c' values; the DNA content of red blood cell nuclei (2c) in Anurans ranges from 2-3$\mu\mu$g in the genera Scaphiopus and Pseudis

to about 17μμg in Rana catesbiana (Goin, Goin & Bachmann 1968 and Goin & Goin 1968), and the ranges in Urodeles is even greater, from 20μμg in Desmognathus monticola (Goin et al. 1968) up to 168μμg in Amphiuma (Commoner 1964).

The three species selected for this study were two Anurans, Xenopus laevis and Bufo bufo and one Urodele, Batrachoseps attenuatus. These particular animals were chosen for the following reasons. X.laevis, a widely used laboratory animal, and B.bufo, an indigenous species, were available in large numbers; furthermore, the 'c' value for Xenopus is well documented and there was reason to believe that B.bufo cells contained about twice as much DNA as X.laevis cells (Browne 1968). B. attenuatus was selected because it supposedly had twice as much DNA per cell as T.vulgaris. (calculated from Browne 1968; and Table 7.2 in Swanson, Merz & Young, 1967).

At the outset of this study it was assumed that in the species selected the physicochemical processes involved in gametogenesis should be subject to the same conditions of temperature, lighting and nutrition. It will be shown below that this assumption was unjustified and led to the wrong approach to the problems involved.

MATERIALS AND METHODS

Mature males of Xenopus laevis and Bufo bufo were obtained through L. Haig, Newdigate, Surrey. Male and female Batrachoseps attenuatus were collected in Southern Oregon or Northern California by Dr J. Kezer of the

University of Oregon. Male and female Triturus vulgaris were collected from ponds on Tentsmuir Point, Fife.

All the animals were kept at 15 ± 1°C under constant illumination. Xenopus and Bufo were kept in large stone tanks, 6-10 animals per tank, and fed twice weekly with earthworms; Xenopus were provided with beef liver occasionally. The Xenopus tanks contained 2in. of water and a piece of corrugated asbestos under which the animals could shelter. The Bufo tanks contained a few inches of damp moss and leaf litter. Batrachoseps were kept in plastic boxes provided with a floor covering of damp absorbant paper and an opaque object under which the animals could shelter. 15-20 animals were kept in each box and they were fed twice weekly with Drosophila. Triturus vulgaris, used solely for DNA determination, were kept in a tank containing 2in. of water and a stone onto which the newts could climb. They were fed twice weekly with Tubifex.

In Bufo and Xenopus all stages of spermatogenesis are present all the year round. 20 Xenopus were obtained in two batches, one in early August 1967 and one in mid March 1968. 22 Bufo were obtained in 3 batches, one in August 1967, one in May 1968 and the third in August 1968. In contrast B.attenuatus testes undergo a seasonal cycle and spermatogenesis commences in June or July, after the breeding season has finished, and continues throughout the autumn and winter. They are difficult to find in large numbers until late September and the 200 animals used in these studies were collected in 3 batches, in October of successive years and arrived in

this laboratory in November.

Each animal was injected with H^3-thymidine (TRA 61 Batch 87, Specific activity 1.9C/mM) obtained from the Radiochemical Centre, Amersham. A single subcutaneous injection of 20μC was given to every male Bufo and Xenopus. An injection of 10μC was given to every Batrachoseps individual in excess of 0.6g weight, the adult status of smaller animals being in doubt. At various time intervals after administration of H^3-thymidine, commencing at plus (+) 4 hrs. and, in the case of Batrachoseps continuing to + 85 days, animals were anaesthetized and testes removed for fixation. In the case of Batrachoseps, animals were sacrificed until a male was encountered and both testes were removed and fixed as follows; one was placed in Clark's Fluid (3 parts absolute ethanol : 1 part glacial acetic acid) and the other is fixed in Sanfelice's Fluid. Both testes are stored in fixative at $2^{\circ}C$ until required for further examination. In the case of Bufo and Xenopus a single testis was removed through a small ventrolateral incision and fixed in Clark's Fluid. The incision is then closed with two catgut stitches and the animals allowed to recover. In this fashion each Bufo and Xenopus provided testes fixations at two different times after injection of H^3-thymidine.

Testes fixed in Clark's Fluid were used for squash preparations, made in the following manner. The whole Batrachoseps testis, or a large fragment of the Bufo or Xenopus testis, was removed from fixative and transferred into a small volume of 45% acetic acid contained in a solid

watch glass. The testis is then tapped out to dissociate the cells and a small sample of the suspension is then placed on a siliconized coverslip. A little more 45% acetic acid is added, the material spread out, and an albuminized slide lowered over the coverslip. The preparation is inverted, and, a few minutes later, firmly squashed between folds of filter paper, by finger pressure. The efficacy of the squash was checked under phase-contrast optics and after inscribing, the slide was placed on a bed of solid CO_2. The coverslip was levered off when the squashed material was frozen. The squash preparation was then immersed in Clark's Fluid for a few seconds and transferred into 70% ethanol for storage. Immediately before preparation of autoradiographs the squash preparations are transferred to distilled water for two minutes. The water is then drained off and the slides kept in a damp atomosphere before coating with Kodak NTB-2 dipping emulsion diluted 50% with distilled water. After coating the slides are thoroughly air dried and left to expose in light-proof boxes at 2°C for about one month.

After suitable exposure the autoradiographs are developed for $2\frac{1}{2}$ mins. in Kodak D19b at 20°C, rinsed in distilled water, and fixed in Kodak Metafix. After further washing for 10 mins., the film is hardened by immersion in 2% formalin for 5 minutes, then re-washed and rinsed in distilled water. These autoradiographs were stained, through the film, with 0.00375% toluidine blue (G.T. Gurr) in 0.001M Sorenson's phosphate buffer at pH 6.1, for 10 mins, washed in distilled water and air dried. They were

ccvered with N°O coverslips using Euparal (Flatters & Garnett) as the mounting medium.

The Sanfelice fixed testes from Batrachoseps were washed for at least 12 hrs. in running water, dehydrated through a graded series of acetones and embedded in methacrylate. 1μ thick sections were cut longitudinally through the entire length of the testis, and mounted on alcohol cleaned slides. The methacrylate is removed with amyl acetate and the material re-hydrated through an acetone series prior to coating with Kodak NTB-2 emulsion. After suitable exposure these autoradiographs were developed and stained as described above. In order to simplify the identification of spermatogenesis stages in the thin sections one 10μ thick section was cut for every ten 1μ sections. These 10μ sections were not prepared as autoradiographs and were stained with Haidenhain's Iron Haematoxyhin.

Squash preparations and sections were examined in detail with a Zeiss GFL Microscope fitted with bright field optics using transmitted light. Photographs were taken using a Zeiss Photomicroscope with automatic exposure.

Comparative DNA values were determined from the diploid blood cell nuclei in two different ways. The bloodcells of Xenopus, Bufo and Triturus are all nucleated and available in sufficient numbers for the application of the Diphenylamine (DPA) reaction according to the technique described by Dische(1955). As only 5-10% of the blood cells of Batrachoseps

attenuatus are nucleated it is impracticable to obtain sufficient nuclei for chemical determination of DNA content. For this reason the DNA content of the four animals was compared by microphotometry of Feulgen stained blood smears.

The DPA reaction was carried out in the following manner. The pericardial cavity of an anaesthetized animal was exposed by gentle dissection. The heart ventricle is cut and the pericardial cavity immediately flooded with a few drops of 0.01M citric acid. As much as possible of the blood suspension is quickly collected with a Pasteur pipette and transferred to 10ml 0.01M citric acid in a centrifuge tube. The suspension is stirred well to prevent coagulation of the blood, and then centrifuged at 2,000 rpm for 5 minutes in a small MSE table-top centrifuge. The supernatant is discarded and the pellet resuspended in 7-8ml citric acid, stirred and recentrifuged. This procedure is repeated until the supernatant is water clear, and the final pellet of nuclei is resuspended in about 5ml citric acid. The number of nuclei per ml. of suspension is determined by haemocytometry. The DPA reaction mixture is made by dissolving 1gm. recrystallized DPA in 100ml glacial acetic acid (analytical grade). 2.75ml concentrated sulphuric acid were added before use. One volume of blood suspension and 2 volumes of DPA reagent were mixed and heated in a water bath at 100°C for 15 mins. After 30 minutes cooling the optical densities at 595mμ of these solutions were measured using a Beckman spectrophotometer.

Blood smears for microphotometry were prepared on alcohol cleaned

3" x 1" glass slides. In order to reduce to a minimum the variations introduced by differences in optical properties between individual slides each slide carried two blood smears to be compared e.g. Xenopus/Bufo; Bufo/Triturus, and Triturus/Batrachoseps. Immediately after the blood smear is made it is placed in a glass chamber in the vapour of concentrated formaldehyde adjusted to pH 6.8 with phosphate buffer. It is allowed to remain undisturbed for 30 minutes. The smear is then transferred directly to 95% ethanol and hydrated through a graded series of ethanols to distilled water. The preparations were rinsed in distilled water at 60°C for 2 minutes, then hydolysed for exactly 12 minutes in Normal Hydorchloric acid at 60°C, rinsed in distilled water and stained for 60 minutes in Feulgen reagent prepared according to the method described by Swift (1955). After staining, the smears are washed in 3 changes of 'SO_2water' (0.5% potassium metabisulphite in 0.05 NHCl) then in distilled water and dehydrated in freshly distilled tertiary butyl alcohol. After clearing in Xylene, the smears are covered with N°O glass coverslips mounted with immersion oil (ref. index 1.515).

The Feulgen content, in arbitrary units, of the blood cell nuclei is determined by the two wavelength method (Patau 1952) as described by Swift & Rasch (1956) using the Zeiss Photometer attachment on a Zeiss photomicroscope. A X25 Planapo objective with an Optovar factor of X1.25 was used throughout the analyses together with a bright field condenser of numerical aperture 1.4. The photometer eyepiece and apertures were changed to accommodate the various nuclear sizes. Details are given in the relevant tables.

OBSERVATIONS

As the events of spermatogenesis are morphologically different in the species examined they will be dealt with separately.

Batrachoseps attenuatus

The haploid number of chromosomes is 13; the meiotic chromosomes are large and well defined and the onset of each meiotic phase is easily identifiable in squash preparations. The meiotic stages are arranged linearly along the length of the testis and longitudinal sections provide a means of checking the data presented by squash preparations.

At the time of arrival in the laboratory (November) the testes usually do not contain spermatogonia although in some individuals, examined within a few days of arrival the mitotic division immediately prior to premeiotic 'S' phase is visible. In squash preparations from testes fixed 4 hrs. after injection of H^3-thymidine, 'S' phase spermatocytes are heavily labelled. Early leptotene cells show localized labelling (Fig. 64 & 65). The patches of label in leptotene cells represents the late replicating regions of the chromosomes and later will be seen to correspond to the condensed chromatin regions immediately adjacent to the centromeres. This 'heterochromatin' is invariably fused to form the 'chromocentre' which is the last region to label with H^3-thymidine (Fig. 64). As in T.vulgaris this chromocentre both starts and finishes replication later than the rest of the chromosomal DNA. Fig. 63 shows a labelled early 'S' phase spermatocyte in which the chromocentre is almost unlabelled i.e. it has

barely started replication.

Labelled chromosomes just commencing synapsis, (i.e. entering the zygotene stage), first appear in fixations made 14 or 15 days after H^3-thymidine injection. Synapsis commences with the chromosome ends, at the opposite pole of the nucleus to the chromocentre (Fig. 66 & 67) and proceeds towards the centromeres. Figure 68 shows a labelled cell, in which synapsis is almost complete, from a preparation made + 39 days after H^3-thymidine injection. During the process of synapsis, the chromocentre begins to break up. It splits first of all into two fractions, which may persist almost to the completion of synapsis where they undergo a further subdivision to give 8 or 9 small chromocentres (not 13 as might have been expected). Figures 69 & 70 illustrate pachytene cells with the multichromocentre condition. Labelled pachytenes are first encountered at + 39 days (Fig. 71 & 72). It can be clearly seen that the silver grains are located over the chromocentres.

Observations beyond + 40 days are subject to certain considerations. It was found that B.attenuatus is not a good laboratory animal for long-term experiments. After 5-6 weeks of laboratory conditions the animals ceased to feed and began to degenerate slowly. It is very difficult to keep B.attenuatus for more than 80 days and by this time the animals are in very poor condition and dying in large numbers. Twenty-one individuals survived to give fixations between 80-85 days after H^3-thymidine injection and of these only 6 were male. Cells labelled in late 'S' phase had not

yet reached 1st metaphase in these animals. In one series, labelled diplotenes (Fig. 73 & 74) were observed in testes fixed 83 days after injection but not in fixations made after 81 or 85 days. In the second series the most advanced labelled cells were in late pachytene 85 days after injection. While scanning the squash preparation made at + 75 days and more it became apparent that the number of cells in division was lower than would be expected. In extreme cases there were many late pachytene nuclei and many developing spermatids but no diplotenes, metaphases or anaphases, nor 2nd meiotic divisions. This observation suggests that meiosis has ceased at the completion of prophase.

Fortunately some indication of the relative duration of the meiotic stages can be obtained from testis sections by counting the number of cysts in each meiotic stage, and relating these figures to a stage of known duration e.g. zygotene. Since late replication continues just into early leptotene and there is no G_2 phase to be accounted for, it is also possible to estimate the duration of 'S' phase in a similar way. The onset of 'S' phase is determined by locating the earliest labelled interphase spermatocyte. Ideally a testis suitable for analysis should contain cells in spermatocyte 'S' phase at one end of the testis and meiotic divisions at the other end. Such testes are very rare. Where 'S' cells are present the testis does not usually contain cells beyond mid-pachytene, and in testes containing meiotic divisions leptotene and pre-leptotene stages are rare (Figs. 75 & 76). Of the testes fixed within 15 days of arrival in the

Animal No.	Time of Fixation	CYST ANALYSIS				Estimated 'S' (days)	Estimated Pachytene (days)
		s/lept.	zygotene.	pachytene.	d/m.		
Bat. 1	+ 4h.	0	16	25	1	-	39.1
Bat. 2	+ 1 day	0	23	39	1	-	42.4
Bat. 3	+ 2 "	14	13	26	1	11-12	50.0
Bat. 5	+ 4 "	0	19	35	1	-	46.2
Bat. 6	+ 5 "	0	10	21	1	-	52.5
Bat. 7	+ 6 "	10	10	15	1	10-11	37.5
Bat. 8	+ 7 "	0	13	27	1	-	51.8
Bat.16	+15 "	6	6	0	0	10-11	-

Mean 45.6 ± 2.3

TABLE I

Cyst analysis of the testes from 8 individual *B. attenuatus*.

laboratory eight animals showed a sufficient range of cell division stages for the duration of pachytene to be estimated but only three individuals provided similar data for pre-meiotic 'S' phase. These data are presented in Table I. It can be seen that the mean estimated value for pachytene duration is 45-46 days which would give a completed prophase time of about 85 days. (This is in good agreement with the one animal yielding labelled diplotenes at + 83 days in squash preparations.)

There are two reasons for believing that this value may be too large. First, the cells of each cyst in the testis do not all belong to exactly the same stage. The cells nearer the mid-line of the testis lag about 1-2 days behind the cells near the outside. This means that at the time of transition from stage to stage of meiosis the cyst contains cells in two different phases. Such cysts were allotted to the category represented by the majority of cells contained within them. Secondly, squash preparations show the onset of pachytene to occur 39 days after injection. Since it is at about this time that these animals cease to feed it is possible that spermatogenesis may already have been disturbed and that the figure for zygotene duration is itself a little exaggerated. Both these errors would give rise to an exaggerated estimate of prophase duration. It seems certain, nevertheless, that meiotic prophase in Batrachoseps is of between 80 and 85 day duration.

The long pachytene stage can be conveniently divided into two phases. The first is a growth phase. On completion of synapsis the chromosomes are

condensed well-defined structures (Figs 68-72). During early pachytene the chromosomes elongate and later pachytene chromosomes have a lampbrush-like appearance. Throughout pachytene the 'bouquet' orientation is maintained.

It is also to be inferred from sectioned material that diplotene and metaphase are of very short duration, both being completed within 2 days. Both division stages are always found in the same cyst of the testis section, frequently together with late pachytene cells (Fig. 76). The in vitro observations of Seto, Kezer, and Pomerat (1969) support the belief that diplotene and metaphase are of short duration, but in vivo they are probably longer than the 8-9 hours reported in that study.

The estimated 'S' phase duration of about 11 days (Table I) is less reliable than the pachytene estimates. To begin with the number of animals with testes containing early spermatocytes in November is small, so small that these individuals must be regarded at least as unusual and perhaps abnormal. Secondly, the absence of a G_2 phase makes the definition of 'S' phase termination and the onset of leptotene a difficult task. This factor will tend to exaggerate the length of 'S' phase although the maximum over estimate will probably not exceed 2 days. It is perhaps more realistic therefore to regard pre-meiotic 'S' phase as lasting more than 8 days but less than 11 days.

Bufo bufo

The haploid number is 11 but the meiotic chromosomes are very small

making accurate detailed analysis of the individual prophase stages impossible. In the testis there is no linear arrangement of meiotic stages, each testis cyst of cells surrounds a seminiferous tubule and all the stages of spermatogenesis are present in each cyst, development taking place from the periphery of the cyst in towards the tubule. The tails of developing sperm project into the tubule, (Champy 1913). The cells in each cyst are too closely packed for an analysis of testis sections, similar to that described for Batrachoseps, to be made.

In fixations made 4 hrs. after injection of H^3-thymidine squash preparations show that spermatocyte 'S' phase cells and cells in early leptotene are lightly but uniformly labelled (Fig. 77). There is no evidence for a late labelling pattern such as that found in Urodeles.

Although the cells are too small to accurately identify the precise beginning of synapsis it is clear that the process is under way in labelled cells + 6 days after injection (Fig. 78 and 79), and + 20 days after injection cells have almost completed synapsis (Fig. 80 and 81).

The identification of the zygotene/pachytene transition is confused by the failure of the synapsed bivalents to separate from one another in the bouquet arrangement so clearly demonstrated by Batrachoseps, and also because the condensation of the chromosomes known as synizesis commences early in the pachytene stage (see King 1907). This phenomenon commences with the appearance of a small mass of chromatin to one side of the nucleus which initially is very similar to the meshwork of chromatin representing

the region of unassociated chromosomes in the late synapsis nucleus.

The end product of synizesis is a highly condensed mass of chromatin (Fig. 81 and 82) which eventually breaks up into 11 bivalents at the onset of metaphase (Fig. 83). If preparations are severely squashed the synizetic knot can be disrupted to show diplotene chromosomes (Fig. 82). H^3-thymidine labelling is first found in the synizetic knot 25 days after injection and labelled metaphases are found 2 days later (Fig. 83). Pachytene in Bufo bufo therefore lasts less than 9 days and at no time during pachytene do the chromosomes exhibit a lampbrush configuration.

It has not been possible to obtain any indication of the duration of 'S' phase. The chromatids are too small for accurate identification at the 2nd meiotic division and it is therefore not possible to identify cells that were labelled during the 'S' phase of the last spermatogonial mitosis (see Callan 1968).

Xenopus laevis

The haploid number is 18 and the meiotic chromosomes are smaller than in Bufo. The same difficulties of identification of meiotic stages are therefore also met here. As described for Bufo the testis is composed of tubules with associated cysts and analysis of testis sections is also impossible.

In squash preparations made from testes fixed + 4 hrs. after H^3-thymidine injection premeiotic 'S' phase cells and early leptotene cells are uniformly labelled (Fig. 84). There is no indication of a late

Experiment 1		Optical density at 595mμ					Mean	Estimated 2c value (μμg)
Species	No. Nuclei per ml. ($x10^6$)	1	2	3	4	5		
X.laevis	31.88 ± 1.5%	0.575	0.540	0.595	0.560	0.570	0.568	6.3*
B.bufo	21.32 ± 1.8%	0.695	0.675	0.710	0.680	0.700	0.692	11.46
T.vulgaris	6.64 ± 3%	1.15	1.05	1.15	1.20	1.20	1.15	61.24
Experiment 2								
X.laevis	36.20 ± 1.5%	0.740	0.710	0.755	0.725	0.770	0.740	6.3*
B.bufo	13.52 ± 3%	0.565	0.530	0.560	0.550	0.555	0.552	12.6
T.vulgaris	2.45 ± 1.7%	0.500	0.490	0.525	0.505	0.485	0.501	63.0

TABLE II

DNA determination using the Diphenylamine reaction.

(* figure taken from Dawid (1965) and Wallace & Birnstiel (1966))

labelling pattern. Labelled early zygotene chromosomes (Fig. 85) are first encountered in fixations made + 6 days after injection and labelled pachytenes found 11 or 12 days later (Fig. 86).

During pachytene the chromosomes form a synizetic knot which breaks up into 18 bivalents at metaphase (Fig. 88). The first appearance of H^3-thymidine in squashed diplotenes was recorded 42 days after injection (Fig. 87) but there are several factors which suggest that this is an abnormally long duration for meiotic prophase in Xenopus. They are discussed later.

Preparations made + 45 days after injection reveal that the most advanced labelled cell is still in diplotene; no labelled metaphases were ever encountered and observations were not continued further.

No estimate of pre-meiotic 'S' phase duration could be obtained.

DNA determination

The results of the DNA determinations are all given in arbitrary units (a.u.) and hence give only the relative DNA values of the species compared.

In each of the two diphenylamine experiments blood from freshly killed animals was used. The DPA was precipitated twice from 96% ethanol and oven dried at 35°C immediately prior to use. Five 1ml. aliquots of the suspension of blood cell nuclei were mixed with 2ml. DPA reaction mixture. The optical densities of these samples was measured at 595mμ. The results are presented in Table II. As controls blank samples of DPA reagent and

0.01M Citric acid were used. This mixture shows no absorbtion at 595mμ.

Feulgen photometry

A random arrangement of pigment molecules throughout a regularly shaped body is the ideal for comparative photometry. Blood cell nuclei only approximate to these conditions; they tend towards oblate spheroids but the chromatin is not evenly distributed throughout the nucleus. They must therefore be regarded as irregular shapes and the Feulgen staining compared using the two wavelength method as described by Patau (1952). This method compares the ratios of the extinction (E) values for the two wavelengths selected. Errors caused by irregular dye distribution are revealed in dissimilar ratios and can be corrected.

The two wavelengths, λ_1 and λ_2, are selected such that $2E_1 = E_2$, where $E_1 = \log \frac{To}{Ts}$ at λ_1 and $E_2 = \log \frac{To}{Ts}$ at λ_2. (To is the intensity of the light transmitted by background material and Ts is that transmitted by the specimen). These wavelengths are chosen from the extinction values at different wavelengths given by a selected nucleus. The nucleus selected is one which most closely approaches the ideal i.e. it is regular in shape and evenly stained. This procedure was carried out for each microscope slide examined. The total amount of dye in the measured area (A), regardless of its distribution, is given by $M = KAL_1D$. Here, $K = 1/e$, where e_1 is the extinction coefficient at λ_1, and was disregarded in these relative determinations. From the light transmissions, T_1 and T_2 (at wavelengths λ_1, and λ_2) L_1 and L_2 were computed such that $L_1 = 1 - T_1$ and $L_2 = 1 - T_2$.

TABLE III DNA content of erythrocyte nuclei determined by Feulgen photometry.

Slide No.	Species	λ_1 (mμ)	λ_2 (mμ)	Aperture (A) (μ^2)	No. Nuclei scanned	Dye content (a.u)	'2c' value (mean) μμg
1	X. laevis	480	565	78.5	10	1,110 ± 54	6.3 (see Tabl
	B. bufo			78.5	10	2,314 ± 57	13.13
2	B. bufo	480	565	78.5	10	1,632 ± 116	(13.13)
	T. vulgaris			201	10	8,373 ± 290	67.36
3	T. vulgaris	490	570	201	23	5,706 ± 51	(67.36)
	*B. attenuatus			491	21	16,357 ± 604	193.05
	**B. attenuatus			201	9	11,850 ± 526	139.90
4	X. laevis	465	570	50.27	25	679 ± 12	6.3
	B. bufo			50.27	25	1,664 ± 45	15.43
5	B. bufo	465	570	78.5	22	1,496 ± 22	(15.43)
	T. vulgaris			201	22	7,678 ± 130	79.?
6	T. vulgaris	465	570	201	20	5,353 ± 138	(79.?)
	**B. attenuatus			201	20	9,173 ± 130	135.75

* Includes haemoglobin contamination

** Selected nuclei, devoid of haemoglobin

The ratio of these values L_2/L_1 was calculated and the corresponding value for D, a correction factor for uneven dye distribution calculated from the transmission values at λ_1 and λ_2, found from the appropriate table (Table V, Swift & Rasch 1956).

Patau (1952) has shown that by careful selection and alignment of optics, and by working in a darkened room the total instrument error can be kept to well below 3%. Further error was encountered in the material. In Triturus and Batrachoseps, and to a lesser extent in Bufo, it was impossible to remove all haemoglobin contamination. Because of the large size of these nuclei, it was difficult to ensure that they remain firmly attached to the slide during hydrolysis and staining unless there was some haemoglobin present. Even small amounts of protein contamination contribute to the total light absorbtion. By careful selection of nuclei with a minimum of haemoglobin contamination this source of error was minimized. There was also a source of error in the nucleus itself. Highly condensed nuclei show greater absorbtion values than others that are less compact, although supposedly the nuclei from the same species contain similar quantities of DNA and should bind similar quantities of Fuchsin. In order to reduce this source of error nuclei were subjectively selected such that they were all of similar appearance. This last source of error made it impossible to compare nuclei with vastly different DNA contents e.g. Xenopus with Triturus or Batrachoseps. In practice few nuclei fulfil all the above requirements. The results of two series of observations are presented in Table III.

DISCUSSION

One of the reasons for the selection of Amphibia for use in this study was the differences in the diploid DNA value that existed between related species. The relative determinations of DNA content for the 4 animals considered here are given in Tables I and II. It is immediately apparent that the photometric determinations are higher than those given by the DPA reaction. It is therefore worthwhile considering the accuracy of each of these procedures.

Several modifications of the diphenylamine reaction have been suggested (Burton 1956; Croft & Lubran 1965; and Giles & Myers 1965) but the original procedure described by Dische (1955) has been used here, for the following reasons. Although the above mentioned modifications achieve a better sensitivity i.e. are capable of detecting smaller quantities of DNA than the original technique, they do so by methods that can result in considerable loss of the DNA during the extraction procedures employed. This is especially so in material that contains large amounts of protein (see Burton 1968). Burton also points out that protein contamination may inhibit the colour development in the modified reactions. This is not the case in the original procedure where low pH and the avoidance of alkali in the tissue preparation reduce interference from protein to negligible proportions. Similarly, the loss of DNA from the tissue is reduced to a minimum in the absence of extraction procedures.

There are however, two sources of error in the DPA reaction. The

first is a statistical error in the counting of blood cell nuclei by haemocytometry. This sampling error has been estimated. It has been reduced by carefully agitating the cell suspension to prevent clumping of nuclei during preparation. Errors in this category may result in either an elevated or a depressed estimate. The second error is in the time allowed for development of the colour reaction. This process is time dependent and may differ between species. Errors in this category will generally lead to depressed estimates. Dische (1955) points out that the colour development is generally complete in about 10 minutes and is stable for several hours. It can be concluded therefore that the 15 minutes incubation at 100°C and subsequent 30 minutes cooling time is quite sufficient for maximum development of the colour in this reaction.

If the 2c value for Xenopus laevis is 6.3μμg (Dawid 1965; and Wallace & Birnstiel 1966) the 2c values for B.bufo and T.vulgaris estimated by the DPA reaction are 12.03 and 62.12 μμg respectively.

The sources of error encountered in the microphotometric technique have already been described. The observations made on Slide 3 (Table IV) indicate that, in the case of Batrachoseps attenuatus protein contamination may produce an error of almost 20%. It is therefore probable that the photometric measurements are high.

Because of the impracticability of comparing X.laevis nuclei with T.vulgaris or B.attenuatus nuclei the sampling errors are cumulative such that, using Xenopus as a standard, the Batrachoseps estimates could be as

TABLE IV Comparison of meiotic prophase duration, and '2c' values.

Species	haploid number (n)	Duration of meiotic prophase stages (days)				's'	Approximate '2c' value (μμg)
		Leptotene	Synapsis	Pachytene	Diplot/metap.		
X. laevis	18	4 - 6	11 - 12	(24-26?)	-	-	6.3
B. bufo	11	4 - 6	15 - 16	7 - 8	1 - 2	-	13
**T. vulgaris	12	5	8	4 - 5	1 - 2	9 - 10	65
B. attenuatus	13	14 - 15	24 - 25	45 - 46	1 - 2	10 - 12	130

* see Tables II and III

** data from Callan & Taylor (1968).

much as 20% in error. Bearing in mind the possible error in the photometric determination the 2c values for B.bufo, T.vulgaris and B. attenuatus, are calculated as 14.28, 73.28 and 137.8 μμg respectively. If, in order to avoid the cumulative error, the DNA values determined by the DPA method are used in the photometric calculations the relative 2c values become 14.28, 61.73 and 117.75 μμg respectively.

Of these values the DPA determinations are probably nearer the true value but a little low (Browne & Macgregor, unpublished, found values of 21 and 71 μμg for B.bufo and Triturus vulgaris), and the photometric figures are high. It is nevertheless clear that the ratios of the Anurans (Bufo to Xenopus) and the Urodele (Batrachoseps to Triturus) are approximately 2:1.

Table IV summarizes the available data concerning meiotic prophase in the four animals studied. Does any pattern emerge from these results that might reflect the different DNA contents of these animals?

Dealing first of all with the Anurans it can be seen that leptotene and zygotene are of essentially similar duration, zygotene in Bufo being only some 25% longer than in Xenopus. If there is a considerable difference in prophase duration between these animals it is chiefly apparent during pachytene; Xenopus pachytene is possibly 3 times as long as that of Bufo, although the figure for Xenopus is suspect. At the time when labelled cells were first recorded in diplotene in Xenopus (+ 42 days) labelled synizetic knots had been present for 6-7 days which, in comparison to Bufo seems an abnormally long time. Furthermore the incidence of dividing

spermatocytes is lower than normal in testis squashes made from Xenopus kept at 15°C for 6 weeks. This observation, as in Batrachoseps, suggests that the normal division cycle has been upset and has in fact slowed down. It is probable that 15°C is too cold to maintain Xenopus in normal breeding condition. This hypothesis is supported by the reports of successful breeding colonies of Xenopus which recommend maintaining a temperature of at least 22°C.

The physiological changes underlying a cessation of spermatogenesis in Amphibia would most probably be located in the pituitary. Follicle stimulating hormone (FSH) from the pituitary initiates spermatogenesis and in Amphibia appears essential for the early stages of meiosis (Walton 1963). Later stages of division and spermatid differentiation are not under hormonal control. Failure of, or a decrease in, FSH production would therefore result in meiotic prophase being suspended, or retarded, while allowing cells in division to proceed further in differentiation. This would produce a testis with a low percentage of cells in division in comparison to other meiotic stages and lead to an overestimate of prophase duration.

Pituitary deficiency, apart from interrupting normal gametogenesis, also results in colour changes in Xenopus. Hypophysectomized animals become pale and will darken again upon injection of extracts from the posterior lobe of the pituitary. During the above experiment no blanching of Xenopus was observed that might also suggest that the pituitary was functioning abnormally, but it must be borne in mind that FSH and the melanocyte

stimulating hormone are produced in different regions of the pituitary.

The data presented for the two Urodeles can be regarded as strictly comparable. The initial doubts about the figures obtained from squash preparations of Batrachoseps testes, have been dispelled by the precisely similar estimates derived from sectioned material. It can be seen that there is a considerable difference in prophase duration between these two species, despite the similarity in duration of the pre-meiotic 'S' phase. Leptotene and zygotene phases are both of longer duration in Batrachoseps by a factor of about 3. Nevertheless, the most striking difference is seen in the relative durations of pachytene. Batrachoseps pachytene phase lasts some 45 days in comparison to the 4 to 5 days of Triturus. An astonishing factor of 10 is involved in this case.

The difference between the duration of the leptotene and synapsis phases in Batrachoseps and those in Triturus is perhaps explained, at least in part, by the difference in chromosome size in these species, but Batrachoseps chromosomes are certainly no more than twice as big as those of Triturus, and probably less than that. However, such differences in size certainly will not account for the disparity in pachytene durations, and if the differences between Xenopus and Bufo pachytene duration are genuine, other hypotheses need to be considered.

There is a considerable difference in chromosome number between Xenopus (n = 18) and Bufo (n = 11) but it is difficult to envisage how this could contribute to the extended pachytene of Xenopus. Such a hypothesis

would need to involve a mechanizm of interchromosomal interference, perhaps a rearrangement of chromosomes in order that they occupy specific positions in relation to one another on the metaphase plate. As far as I know there is no evidence for quite such specific orientation of chromosomes although Ockey (1969) presents evidence for, and discusses other reports of, a non-random distribution of chromosomes in colcemid blocked metaphases in human fibroblasts.

The absence of a large difference in chromosome number between Triturus and Batrachoseps, while at the same time exhibiting an even greater disparity in pachytene duration, indicates that some alternative explanation should be sought.

It is generally believed that genetic recombination (crossing over) occurs during pachytene and it is also with pachytene (paired) chromosomes that the synaptinemal complex (Moses 1958) is associated. In a recent review, Moses (1969) has indicated that the synaptinemal complex (SC) is necessary for crossing over (and hence chiasma formation) but is not an essential feature of paired chromosomes. Moses therefore suggests that the SC is involved specifically with crossing over and proposes three ways in which the structure may facilitate effective synapsis as a prelude to successful crossing over. These proposals are "a) to maintain pairing in a fixed state for an extended time, b) to provide a structural framework within which molecular recombination may occur, and c) to segregate recombination DNA from the bulk of chromosomal DNA".

Since the SC becomes associated with paired chromosomes simultaneously with synapsis of those chromosomes it is likely that conditions fulfilling a) and b) above are already established at the onset of pachytene. Any difficulty or complication of the process would result in an extended synapsis duration.

It is also possible that function c) has been fulfilled at the completion of synapsis but one explanation for a prolonged pachytene could be that considerable reorganization of chromosomal material is necessary in order to bring into juxtaposition those small lengths of DNA that will be involved in crossing over. In this context the relative chiasma frequencies will be of interest.

The bivalents of Bufo and Xenopus are unfortunately too small to determine the chiasma frequency but some data is available for Triturus and Batrachoseps. Squash preparations prepared for autoradiography do not allow accurate counting of chiasma but aceto-orcein stained squash preparations made by H.G. Callan in 1955 have permitted estimates of chiasma frequency to be made. The results are presented in Table V. It is immediately apparent that Batrachoseps forms at least twice as many chiasma as does T.vulgaris. If the formation of chiasma is a sequential process, like the process of synapsis then high chiasma frequencies would be associated with ... tend to increase the duration of pachytene. Does this hypothesis withstand closer investigation?

TABLE V

Chiasma frequency in male T.vulgaris and B.attenuatus. Measured from 1st meiotic diplotenes.

Species	Animal No.	No. cells counted	Chiasma number
T.vulgaris	55/3	30	22.77 ± 0.204
	55/5	30	21.44 ± 0.475
	55/7	30	22.77 ± 0.184
	55/8	30	22.60 ± 0.252
	55/9	30	22.10 ± 0.365
B.attenuatus	Single individual	11	48.45 ± 3.44

There is already some evidence that chiasma formation takes place in a linear sequence. Henderson (1963b) analysed the chiasma distribution at diplotene in Schistocerca gregaria and found a linear relationship between the chiasma frequency and chromosome length for the long acrocentric chromosomes, and for those medium length chromosomes with a chiasma frequency above 2 per bivalent. Henderson concluded that chiasma formation usually begins at the distal end of these chromosomes and proceeds towards the centromere. He also found some evidence that medium length chromosomes begin chiasma formation at both ends simultaneously and the process continues towards the centre of the chromosome. Also in support of the contention that chiasma formation is a time sequential operation in further data presented by Henderson (1963a and 1966) concerning depression of chiasma

frequency in S.gregaria by incubation at high temperatures. In those reports Henderson shows that the lowering of chiasma frequency is first detectable after 2-3 days at 40°C and reaches a peak depression some 6 days after the start of treatment. Such a gradual decrease in chiasma frequency is consistant with the above hypothesis and may provide a method whereby it can be experimentally tested.

The chiasma frequency in Batrachoseps kept at normal temperatures could be accurately established using the technique described by Henderson (1963b) and the subsequent effect of elevated temperature upon this chiasma frequency could be studied. The extremely long duration of zygotene/pachytene in Batrachoseps may facilitate the accurate timing of interference with chiasma formation and could reveal the sequential nature of the process. Parchman & Stern (1969) report the suppression of chiasma formation by cyclohexamide and this should be borne in mind as an alternative method for interfering with chiasma formation.

There is another possible explanation for the disparity in pachytene duration. It was pointed out in the Introduction to this section that the pachytene phase in spermactocytes corresponds approximately to the lampbrush phase of oocytes. It is therefore possible that the long duration of pachytene reflects a master/slave gene matching procedure immediately following recombination according to the scheme suggested by Callan & Lloyd (1960) and Callan (1967). If this is indeed the explanation then the extended pachytene of Xenopus appears somewhat anomalous. If there is a

difference in the degree of repetition in the genomes of Bufo and Xenopus it might be expected that Bufo would show the higher value, not Xenopus.

Callan & Taylor (1968) made the suggestion that there may be an alteration, an increase, in the length of replicating units during the 'S' phases, immediately prior to meiosis. They present evidence to show that 'S' phase duration increases with each subsequent mitosis during the division of spermatogonia and reaches a maximum duration at the pre-meiotic 'S' phase. If Callan & Taylor's interpretation of this phenomenon is correct it might be expected that the increased replicon length reflects the length of the repeated sequences in the genome. There are several reasons for believing this not to be the case. First of all the similarity in pre-meiotic 'S' phases duration in Triturus and Batrachoseps argues against it. This is especially true when the indications are that the experimental conditions described above tend to maximize the other differences in the meiotic cell cycle of these two animals. Furthermore, it has been shown that the rate of DNA synthesis may differ considerably within the same species. Church & Robertson (1966) demonstrated significant differences in the rate of larval DNA synthesis in lines of Drosophila melanogaster selected for large or small body size and fast or slow development times. In this case, it is improbable that changes in replicon length occur and it is more likely that the differences in the rate of DNA synthesis reflects the rate at which replicons become available for

duplication. This alternative explanation was also suggested by Callan & Taylor (1968).

However, before conclusions can be drawn from the data presented for Xenopus the above experiments should be repeated at a temperature more suitable for Xenopus. Perhaps the correct procedure would be to compare these animals under conditions where DNA replication and meiosis are proceeding at their maximum rate. Such conditions would need to be empirically determined.

SUMMARY

The time course of male meiotic prophase has been determined for the Amphibians Xenopus laevis, Bufo bufo and Batrachosens attenuatus kept at 15 ± 1°C. Under the conditions of the experiment it is thought that the values for X.laevis are exaggerated.

The durations of leptotene in X.laevis, B.bufo and B.attenuatus are 4-6, 4-6 and 14-15 days respectively. For synapsis they are 11-12, 15-16 and 24-25 days, for pachytene 24-26, 7-8 and 45-46 days, and for diplotene and metaphase combined about 2 days although this last figure is not available for X.laevis.

The duration of pre-meiotic DNA synthesis has been estimated for B.attenuatus and lasts for some 10-12 days.

The DNA content of the diploid blood cell nuclei in X.laevis, B.bufo, B.attenuatus and also in T.vulgaris has been determined. By the diphenylamine reaction this 2c value for B.bufo and T.vulgaris is 12.03 and 62.12 μμg /nucleus respectively if a 2c value of 6.3 μμg is assumed for X.laevis (Dawid 1965; and Wallace & Birnstiel 1966). By microphotometry of Feulgen stained blood cell nuclei the 2c values for B.bufo, T.vulgaris and B. attenuatus were calculated as 14.28, 73.28 and 157.82 μμg. In the cases of B.bufo and T.vulgaris reasons are given for believing the true value to lie between these two estimates. There is also reason to believe that the estimate for B.attenuatus is higher than the true value.

The differences in meiotic prophase duration in these animals is

discussed, and the data presented for B.attenuatus is compared with previously published data for T.vulgaris. It is suggested that the large disparity in pachytene duration in these species may reflect complications in the process of chiasma formation rather than a manifestation of the master/slave gene matching procedure postulated by Callan (1967).

The similarity in 'S' phase duration between T.vulgaris and B.attenuatus is regarded as evidence that the prolonged pre-meiotic replication phase is due to a difference in the rate at which replicons become available for duplication rather than to a difference in replicon length.

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P L A T E S

Except where otherwise indicated the figures on any one plate are shown at the same magnification. The scale is indicated on the first figure of each plate.

PLATE I

Isolated lampbrush chromosomes of T.c.cristatus

Fig. 2: Giant normal loop on chromosome I from an untreated oocyte 0.7mm. diameter.

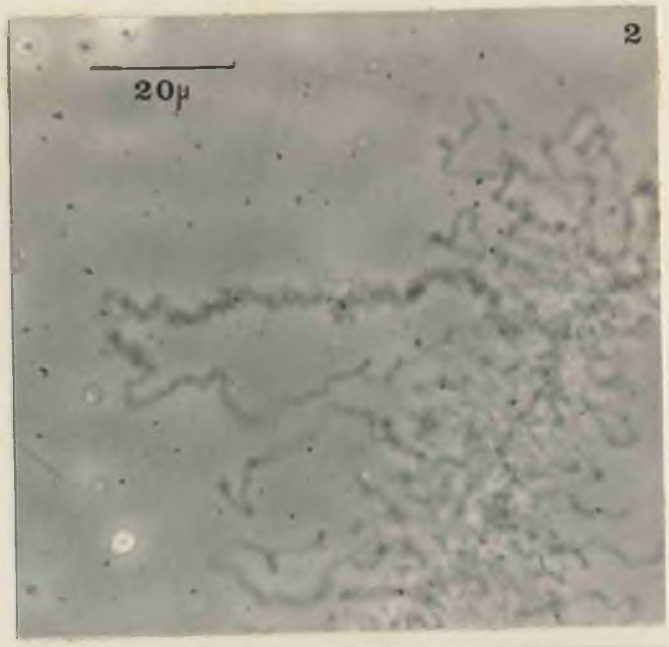
Fig. 3: A pair of giant loops on chromosome I after 4h. treatment in vivo with Actinomycin D (100µg/ml.). Oocyte diameter 0.7mm.

Fig. 4: As fig. 2 but 2 days after Actinomycin D treatment. Oocyte diameter 0.6mm.

Fig. 5: The group of lumpy loops on chromosome II from an untreated oocyte 0.6mm. diameter. Animal S11.

Fig. 6: As fig. 5 after 4h. Actinomycin D treatment in vivo. Oocyte diameter 0.8mm. Animal S11.

Fig. 7: Lumpy loops from animal S11 examined 14 days after Actinomycin D treatment. The mass of lumpy loop material is noticeably increased. Oocyte diameter 0.8mm.



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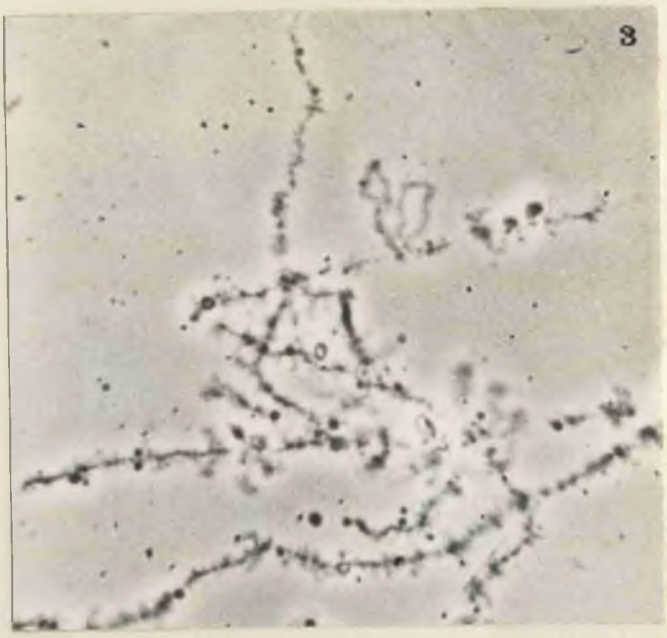

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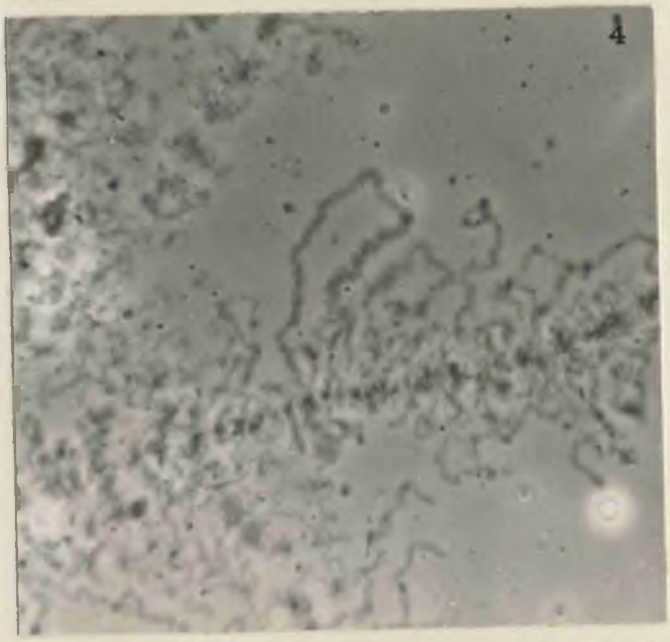

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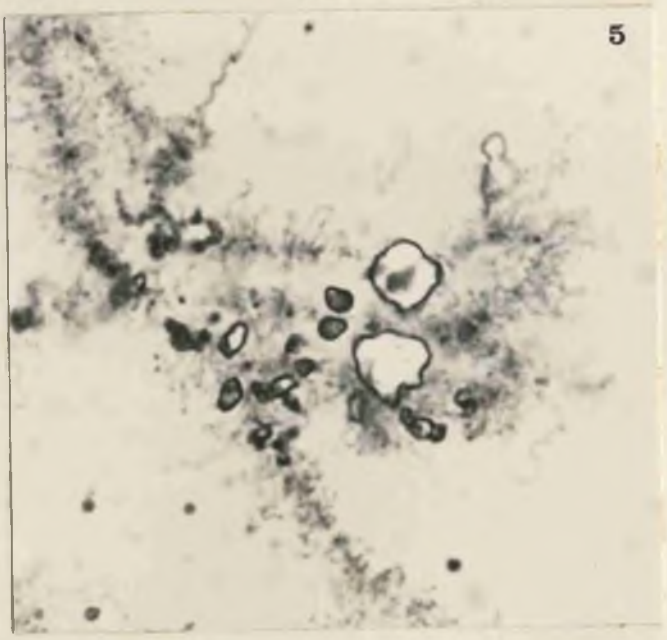

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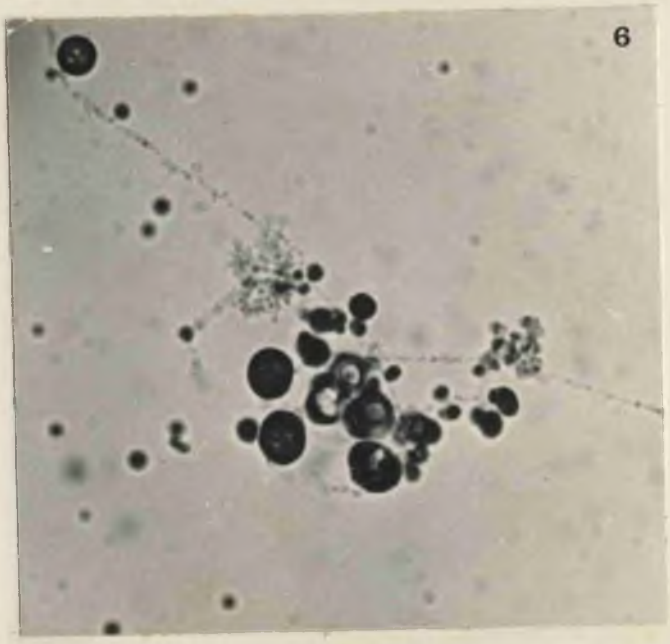

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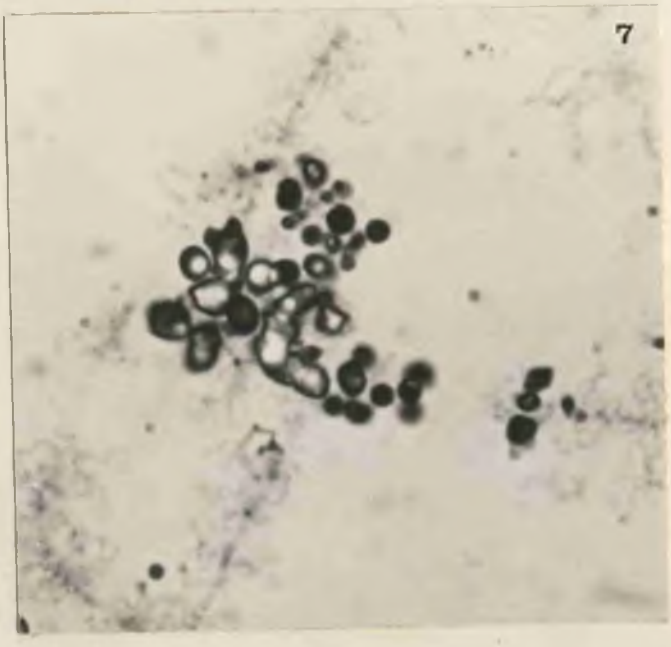

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PLATE II

Isolated lampbrush chromosomes of T.c.cristatus

Fig. 8: The giant fusing loops on chromosome XII from an untreated oocyte 0.7mm. diameter.

Fig. 9: Giant fusing loops after 4h. Actinomycin D treatment in vivo. Oocyte diameter 0.7mm. Note the small contraction in volume.

Fig.10: Giant fusing loops from animal S11 (see figs. 5-7) examined 14 days after Actinomycin D treatment. Oocyte diameter 0.8mm.

Fig.11: Autoradiograph of the giant fusing loops made immediately after the 4h. Actinomycin D treatment in vivo. The animal from which this preparation was made received a subcutaneous injection of 200µC H^3-uridine 12h. before Actinomycin D treatment. The silver grains indicate that recently synthesized RNA is retained during Actinomycin D treatment. Oocyte diameter 0.8mm. Exposure time 60 days.

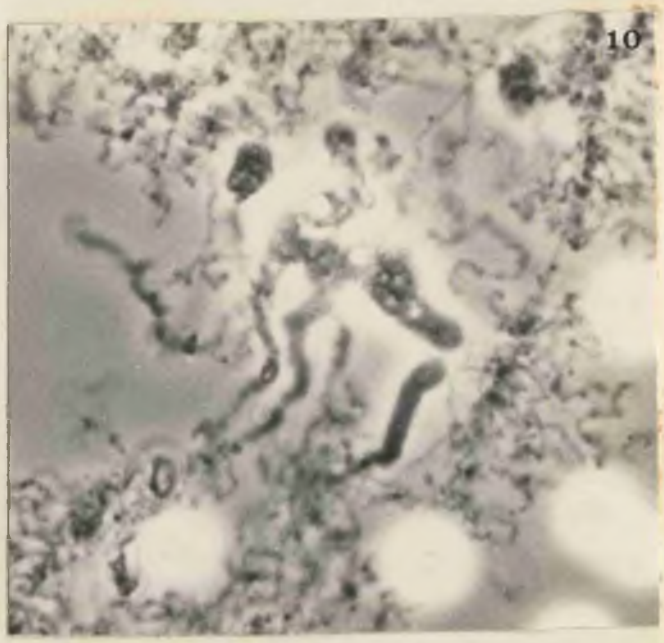
Fig.12: Spheres on chromosome V from an untreated oocyte 0.8mm. diameter.

Fig.13: Spheres after 4h. Actinomycin D treatment. Oocyte diameter 0.8mm.

Fig.14: Spheres 1 day after Actinomycin D treatment. Oocyte diameter 0.9mm.


20μ
8


9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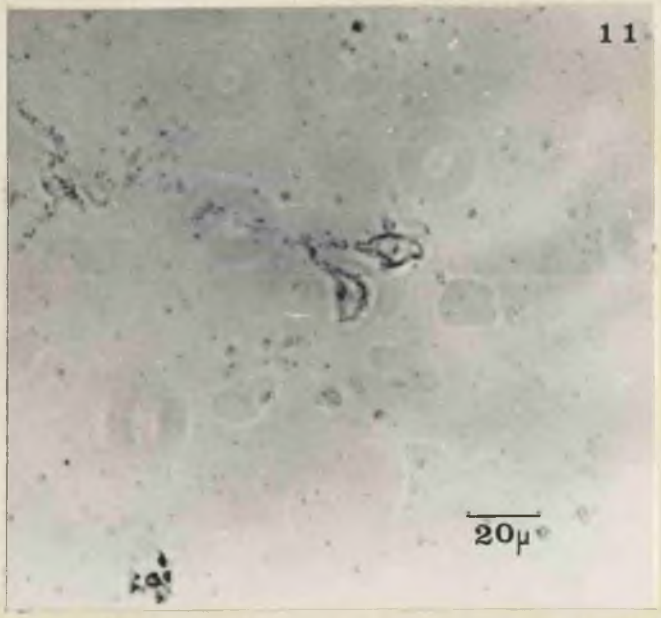
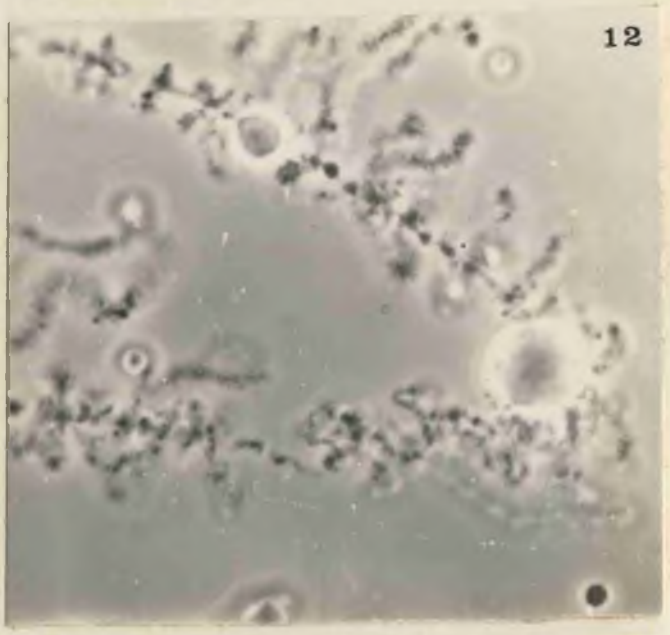

11
20μ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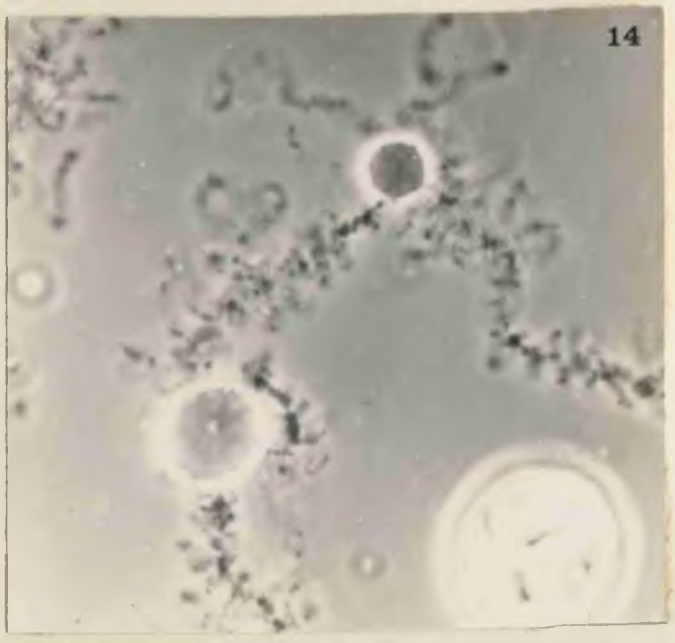
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PLATE III

Peripheral nucleoli; light microscope studies

Fig.15: Autoradiograph of isolated nucleolus from an untreated oocyte 0.7mm. diameter. 12h. availability of H^3-uridine. Exposure time 60 days.

Fig.16: Autoradiograph of isolated nucleolus from an oocyte 0.7mm. diameter sampled immediately after Actinomycin D treatment. Exposure time 60 days. Note the absence of silver grains above the nucleolus.

Fig.17: As figs. 15 and 16 after 1 day of recovery from Actinomycin D poisoning. Note that nucleoli recover the ability to incorporate H^3-uridine. Exposure time 60 days.

Sections through untreated oocytes, to show nucleoli.

Fig.18: Stained with Haidenhain's Haematoxylin.

Fig.19: Stained with Azure B bromide.

Fig.20: Stained with gallocyanin chrome alum.

Fig.21: Viewed under phase-contrast optics.

Sections through oocytes given 4h. treatment in vivo with Actinomycin D.

Figs.22-24: Stained with Haidenhain's Haematoxylin. Note the characteristic crescent shaped zonation of the nucleoli. Fig. 24 shows a clear space between the two regions which may be a form of vacuolation.

Figs.25&26: The zonation seen under phase-contrast optics.

Figs.27&28: The occasional, different form of zonation seen with Azure B bromide as the stain.

Figs.29&30: Nucleoli stained with gallocyanin chrome alum. No zonation is found in these nucleoli.

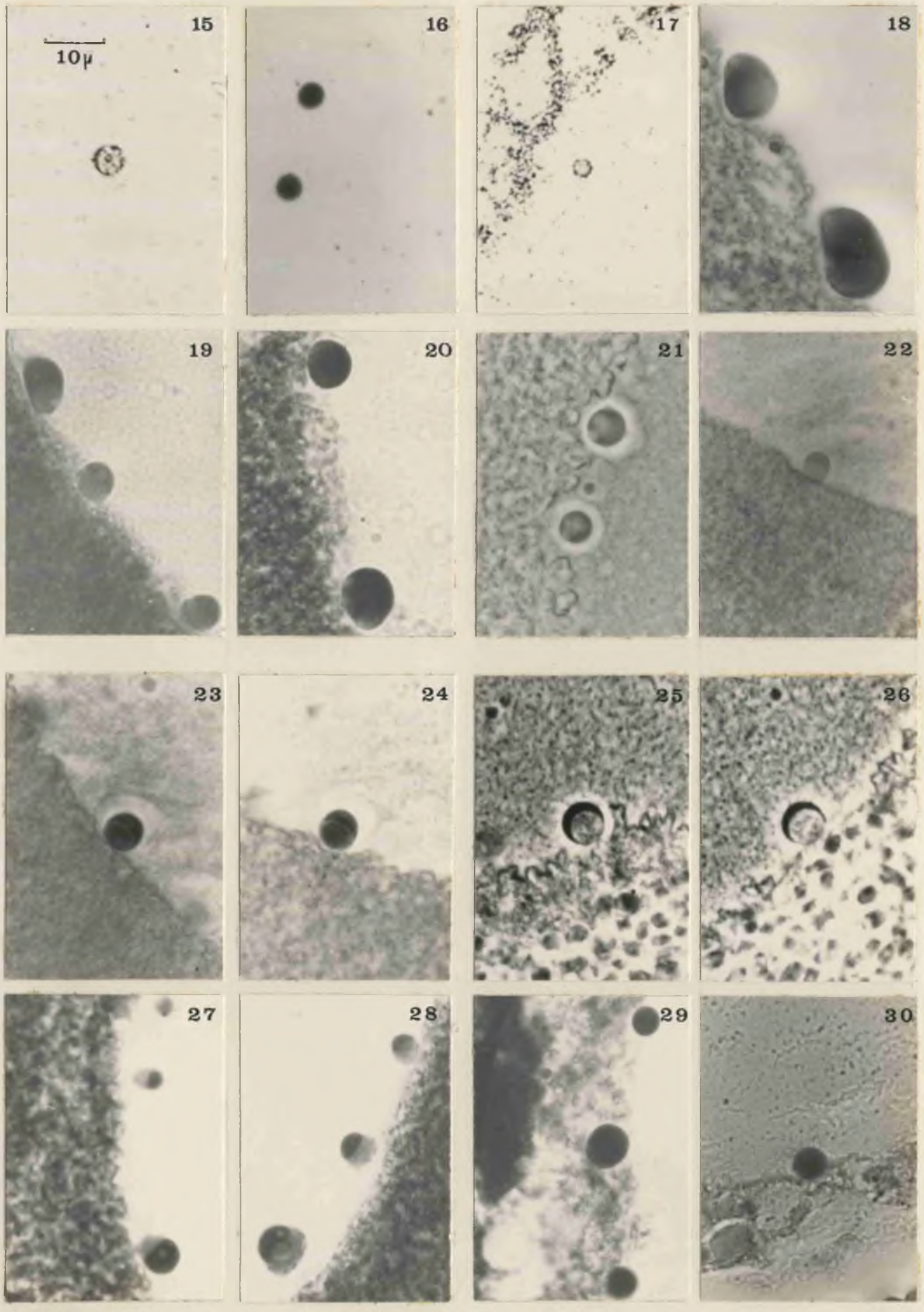
15
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PLATE IV

Peripheral nucleoli: light microscope studies.

Fig.31: Untreated oocyte from Bufo bufo sectioned and stained with gallocyanin chrome alum to demonstrate the non-uniform distribution of RNA in these nucleoli.

Fig.32: As figs. 22-24 but after ribonucleose digestion. The zonation is apparently not due to RNA distribution.

Sections through oocytes sampled 1 day after Actinomycin D treatment.

Figs.33&34; Stained with Haidenhain's Haematoxylin.

Figs.35&36: Viewed under phase-contrast optics.

Autoradiograph of oocyte sections.

Figs.37&38: Untreated oocyte. Exposure time 36 days.

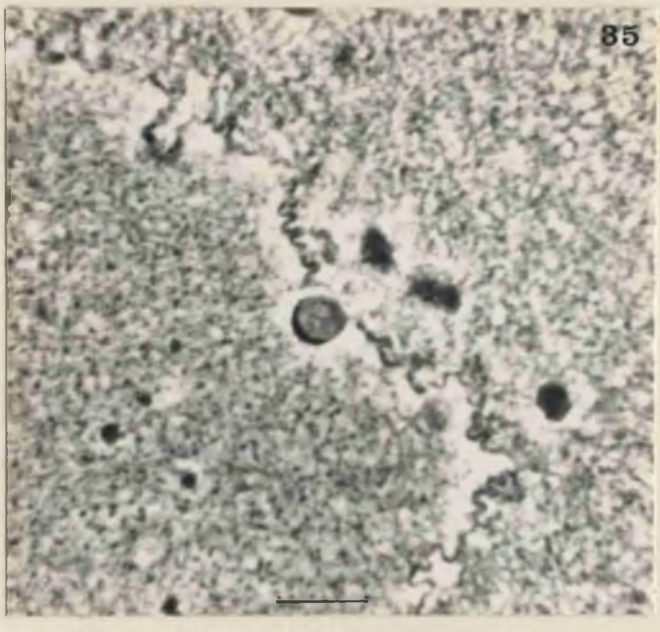
Figs.39&40: Oocyte immediately after 4h. Actinomycin D treatment. Exposure time 36 days.


31
10μ


32


33


34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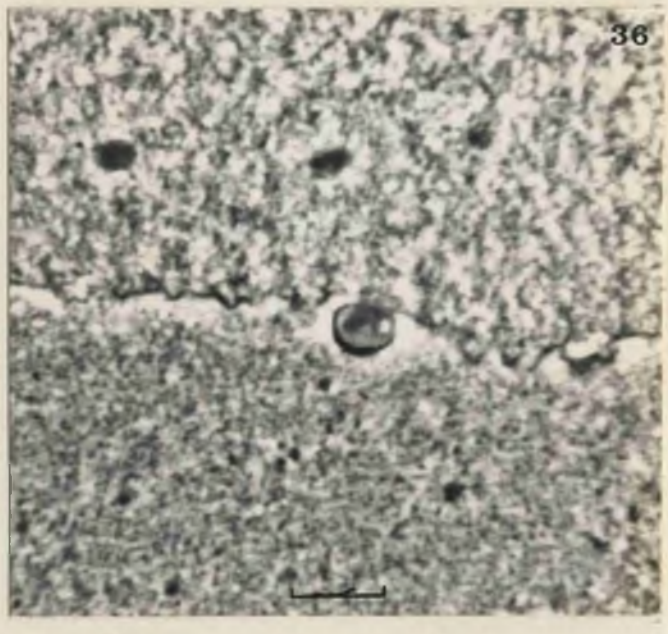
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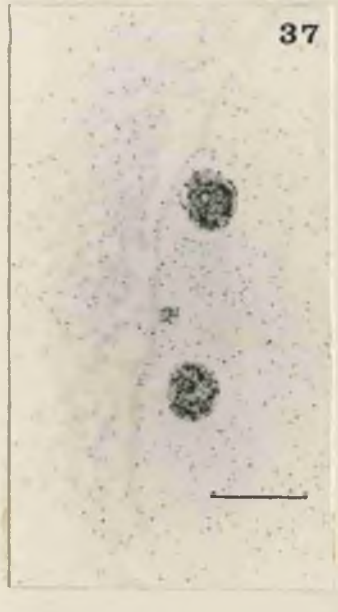
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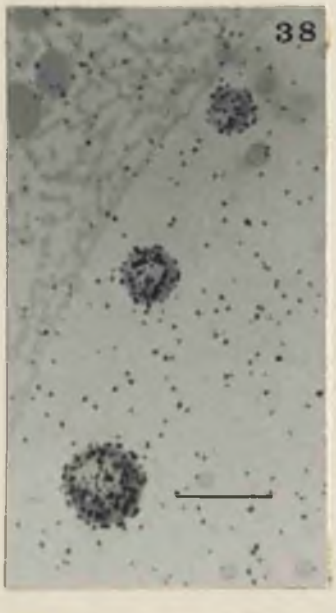
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39

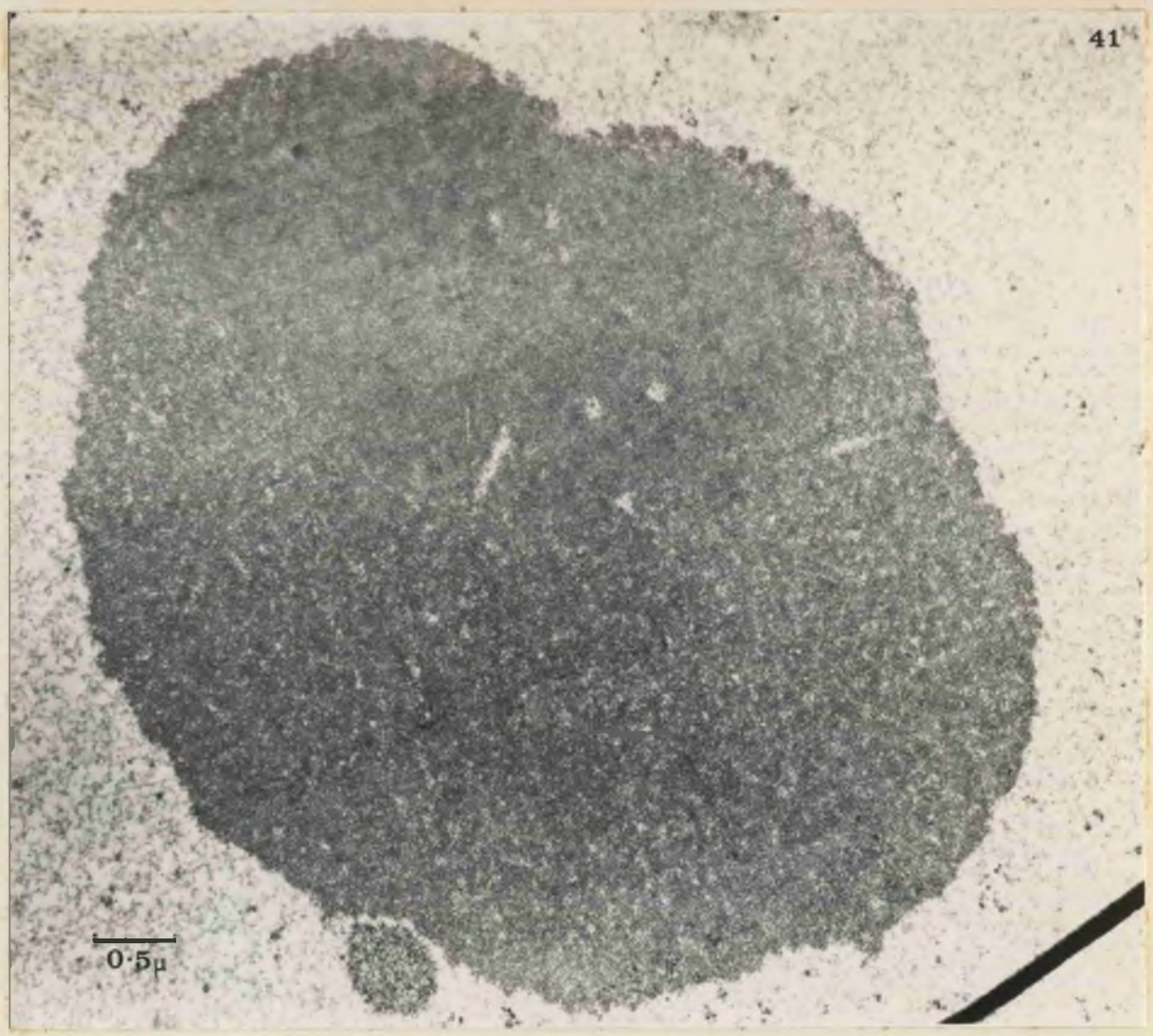

40

PLATE V

Electron microscopy; peripheral nucleoli of untreated oocytes from T.c.carnifex.

Fig.41: A peripheral nucleolus showing a particularly well defined core and cortex. The core, situated in the upper part of the nucleolus, is directed toward the nuclear membrane. It shows a higher concentration of granular, fibrillar and amorphous components than the cortex. Oocyte diameter 1.00mm. x 20,000.

Fig.42: This peripheral nucleolus is more typical of T.cristatus in that no zonation is apparent. Oocyte diameter 0.9mm. x 20,000.


41
0·5μ

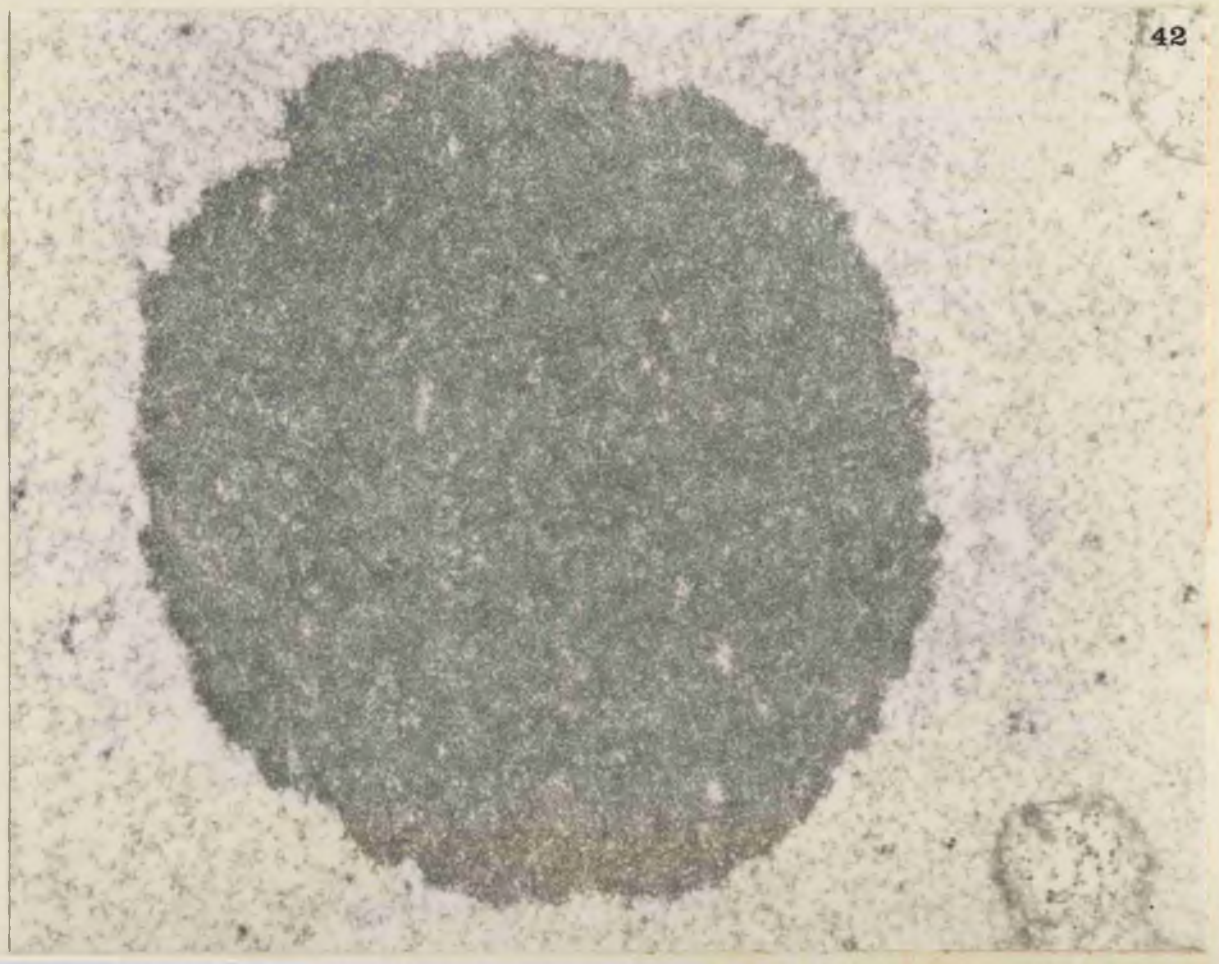

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PLATE VI

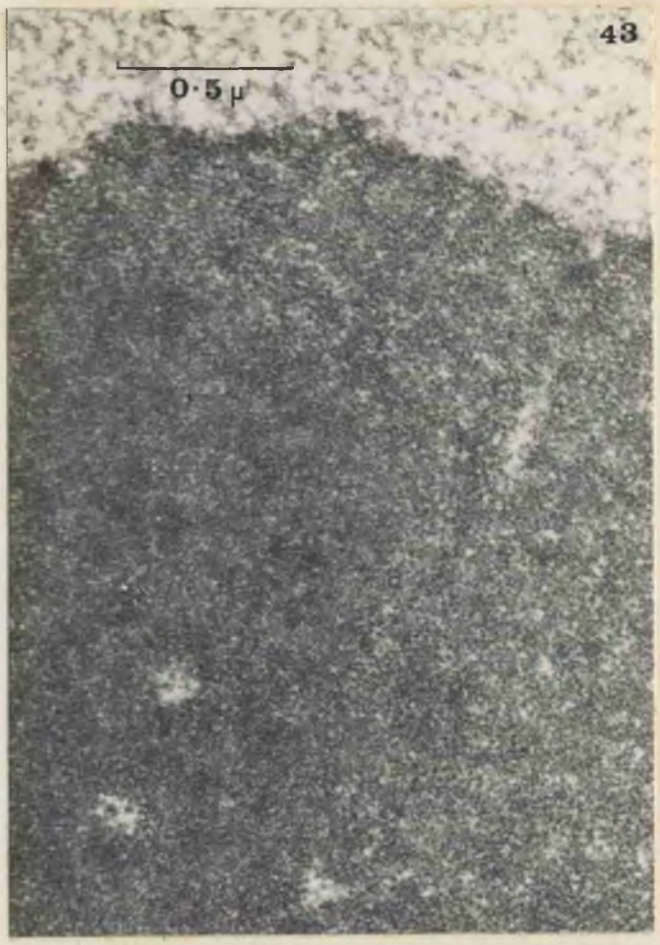
Electron microscopy; peripheral nucleoli and micronucleoli of untreated oocytes.

Fig.43: Part of the nucleolus shown in fig. 41. The difference between core and cortex is well illustrated. x 40,000.

Fig.44: A micronucleolus from a 0.9mm. oocyte x 60,000.

Fig.45: A micronucleolus from a 0.8mm. oocyte x 60,000.

Fig.46: As fig. 44. The apparent continuity of these bodies with the nuclear sap is seen at the top and bottom of this picture. Also clearly illustrated are the granular aggregates and the fibrils (see arrow) which compose these bodies. x 90,000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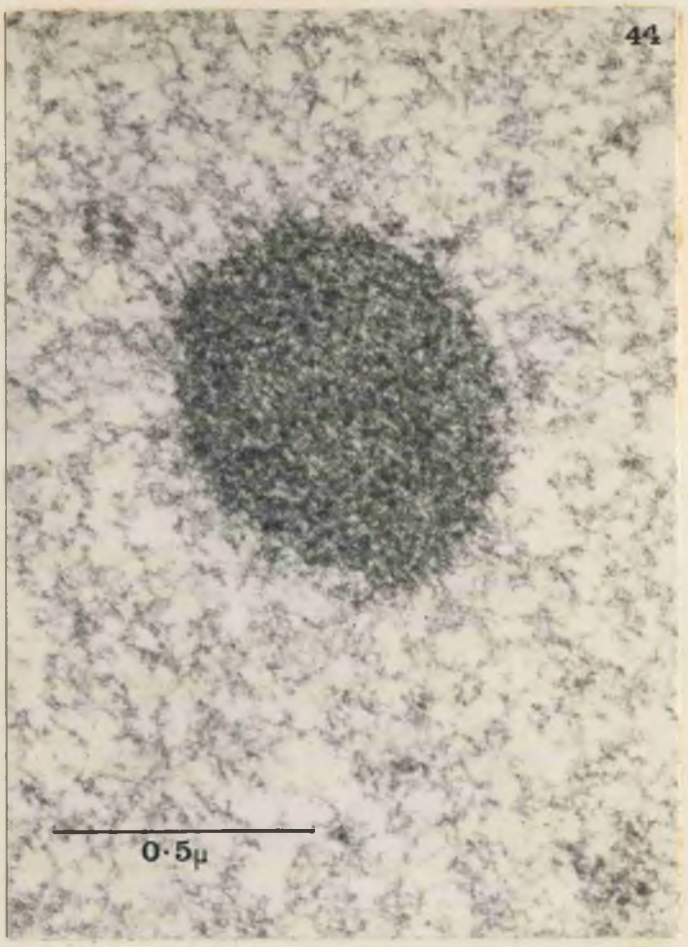
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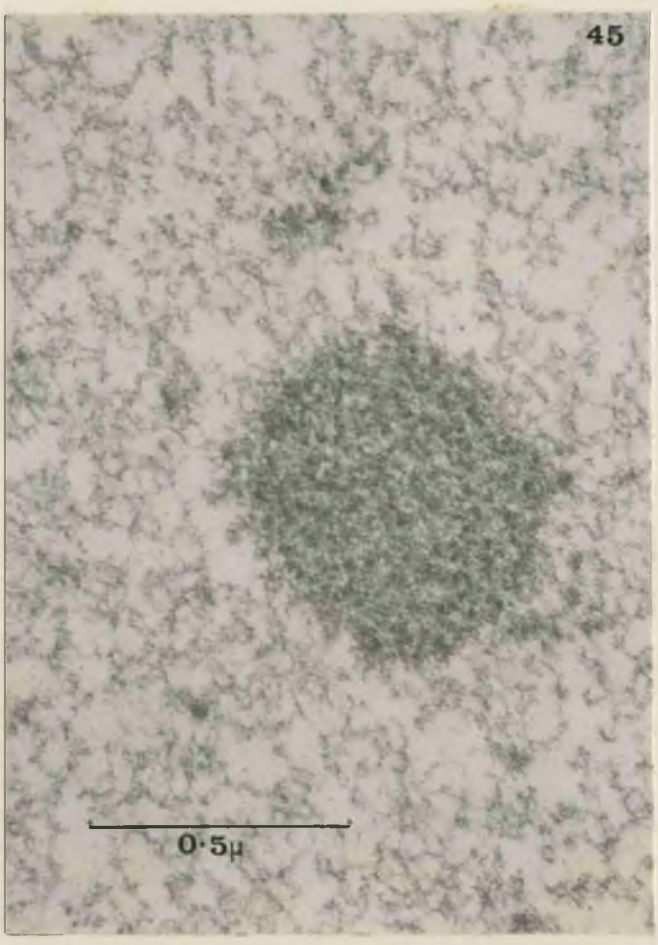
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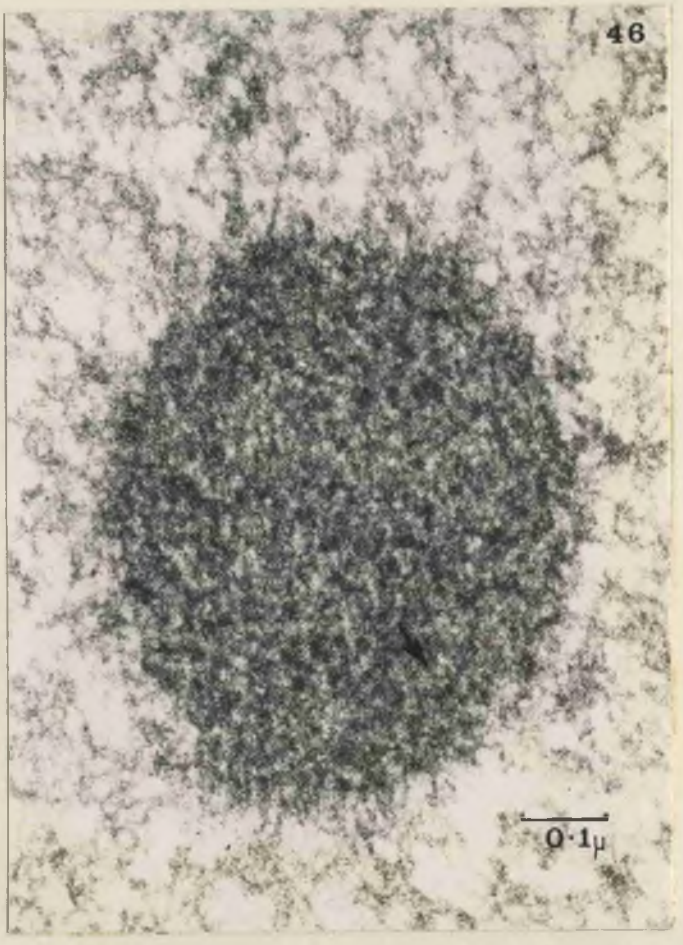
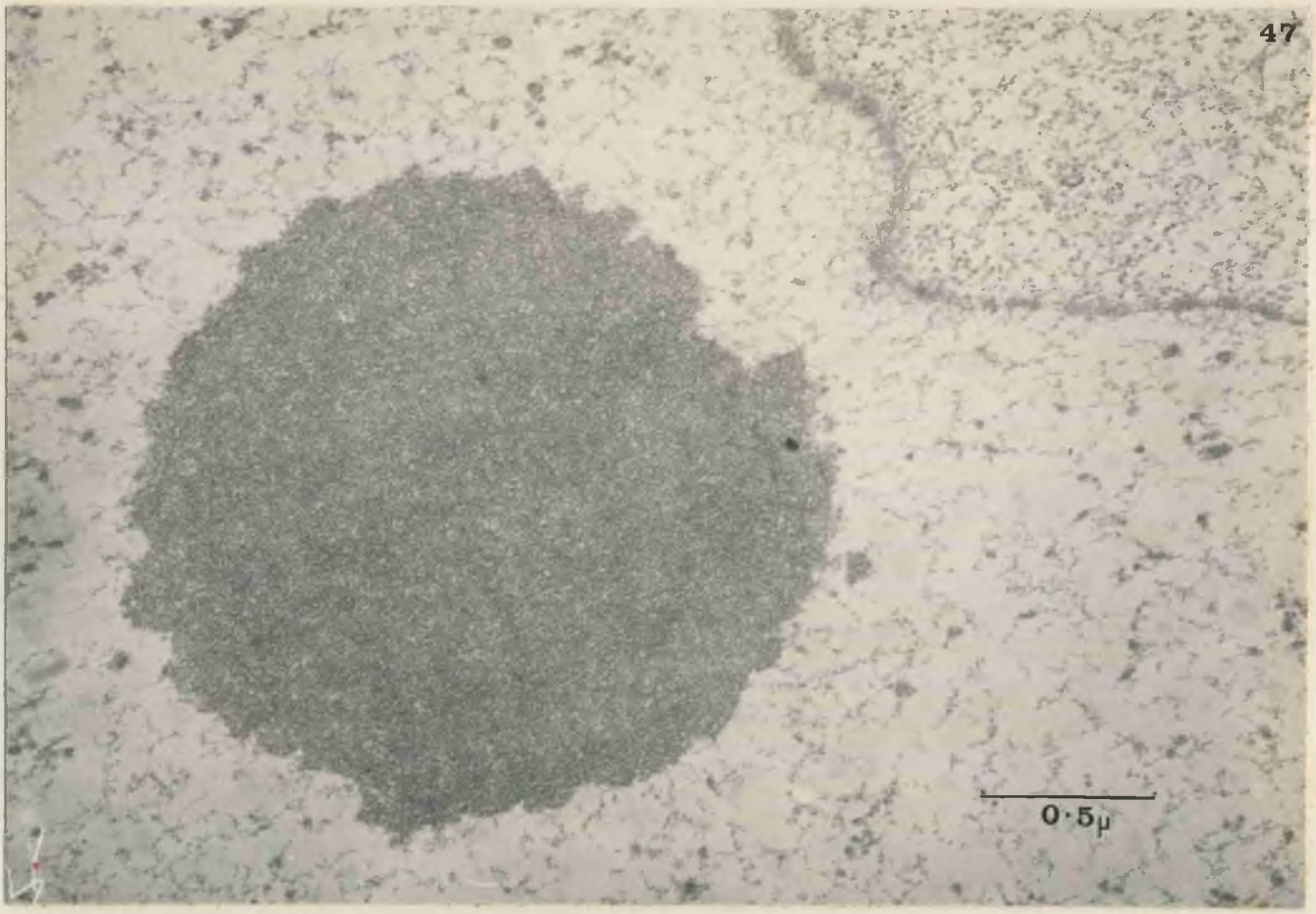

46
0·1μ

PLATE VII

Electron microscopy; peripheral nucleoli from oocytes incubated in 100µg Actinomycin/ml. for 4h.

Figs.47&48: Peripheral nucleoli from a 0.9mm. diameter oocyte. It can be seen that as a result of Actinomycin D poisoning the coarser granular component of the nucleolus has been lost. The nucleoli have shrunk and there has been a reduction in the ribonucleoprotein network in the nuclear sap. (For comparison, see figs. 41 & 42) x 40,000.


47
0·5μ

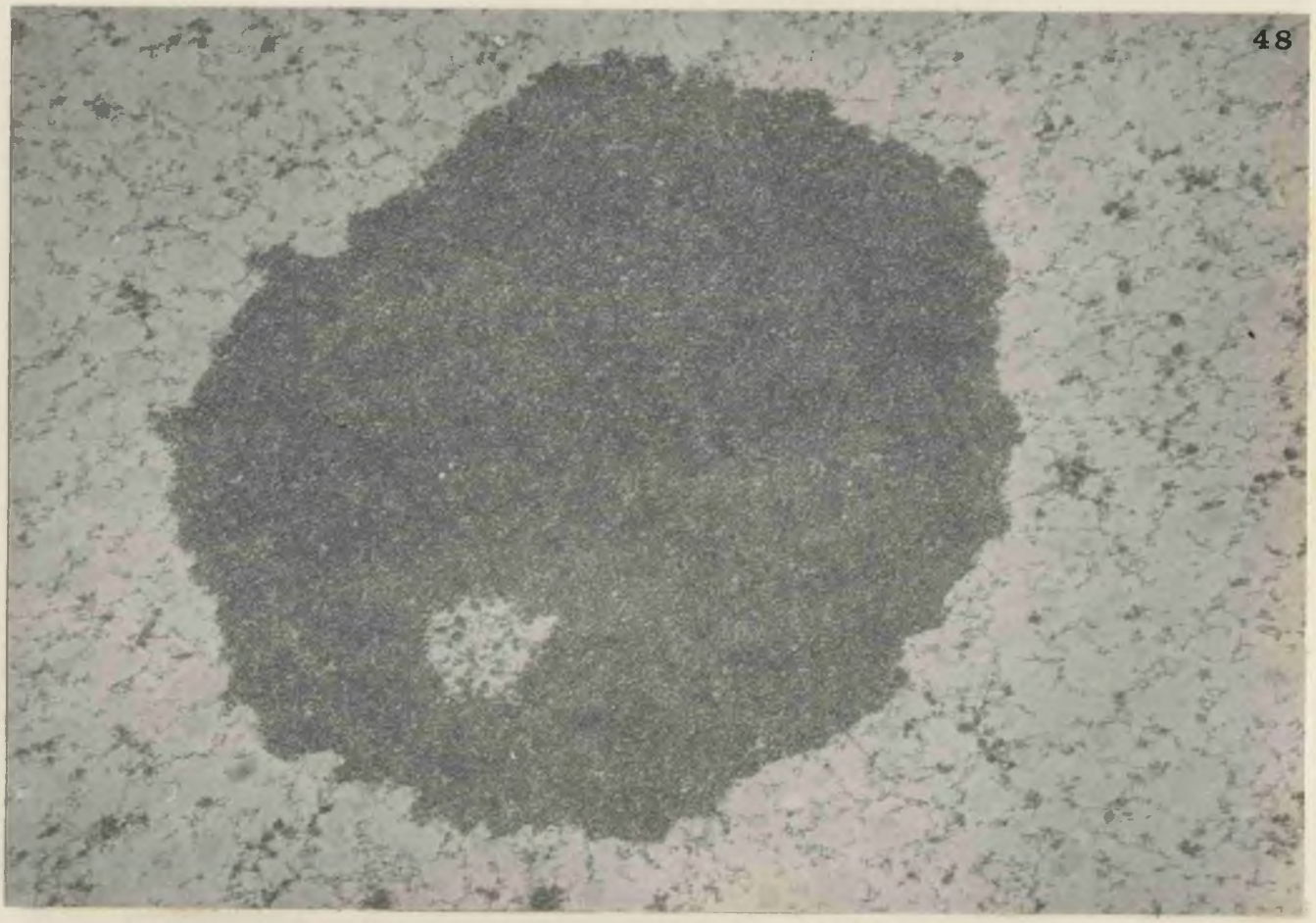

48

PLATE VIII

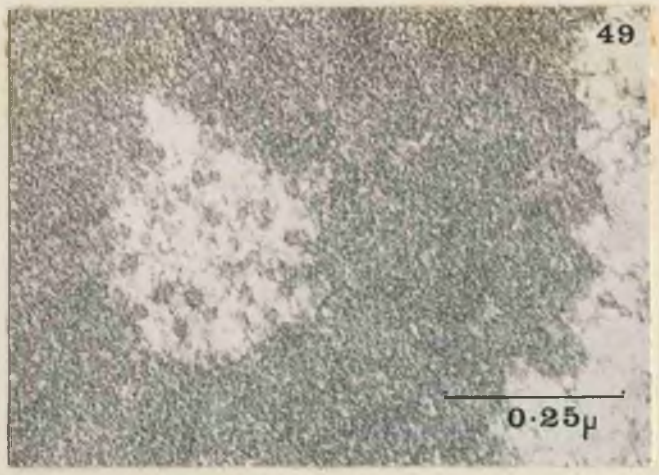
Electron microscopy: peripheral nucleolus, micronucleoli and Lamellar bodies in Actinomycin D treated oocytes.

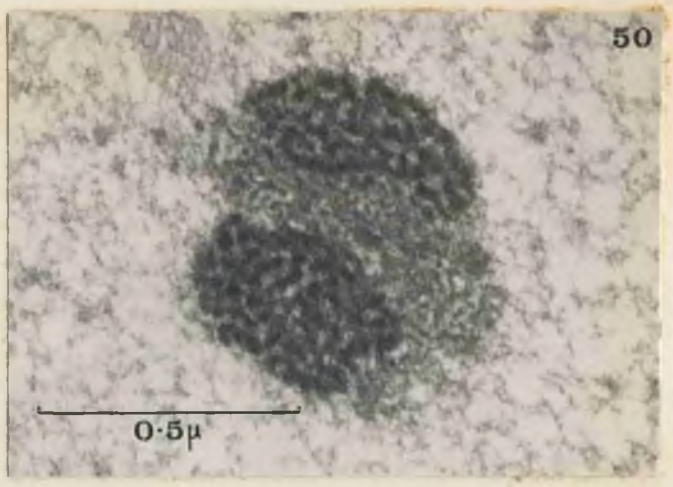
Fig.49: Part of fig. 48 showing fibrils and small granular component. x 80,000

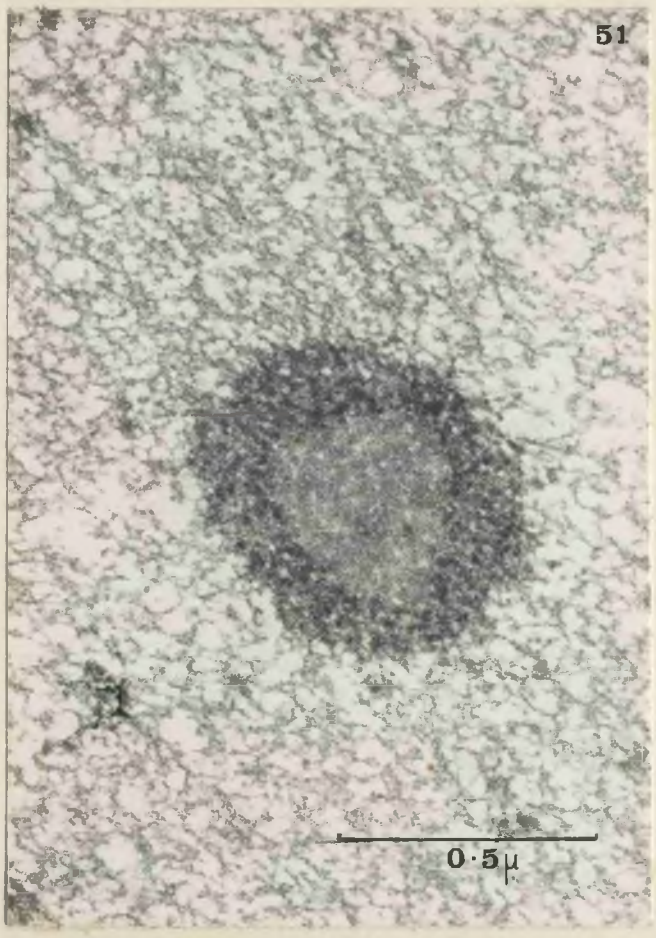
Figs.50&51: Micronucleoli showing clearly the concentration of the granular component at the periphery of these bodies. The continuity between nuclear sap mateiral and the micronucleolar granular component is even more marked than in untreated oocytes, especially in fig. 51. x 60,000

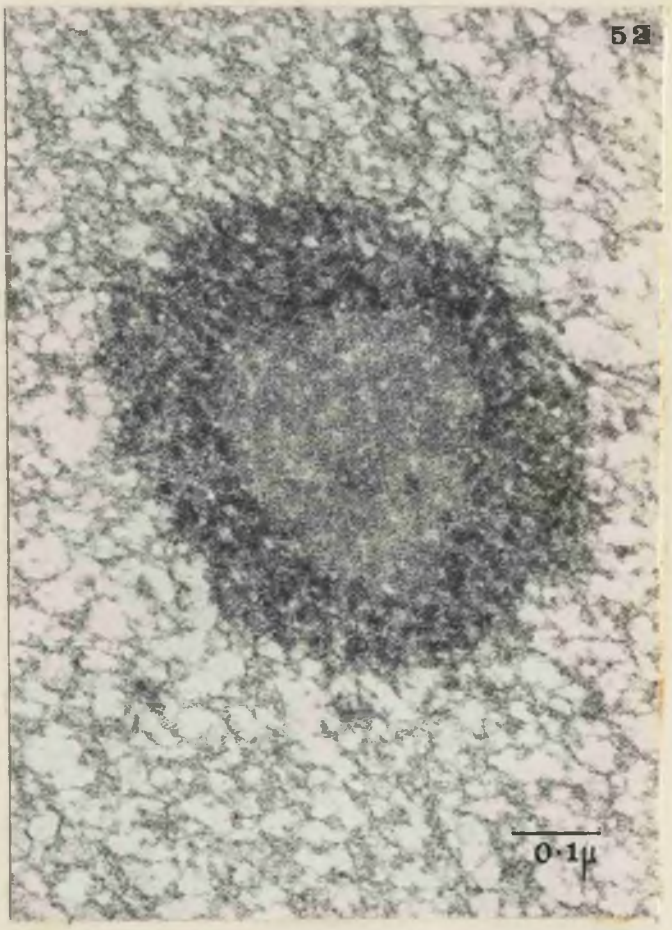
Fig.52: As fig. 51. Fibrillar material is very clearly shown in the centre of this micronucleolus. x 90,000

Fig.53: Longitudinal section through a particularly large example of the lamellar bodies induced by Actinomycin D. This particular body was about 3μ long and o.16μwide. x 60,000


49
0·25μ


50
0·5μ


51
0·5μ


52
0·1μ

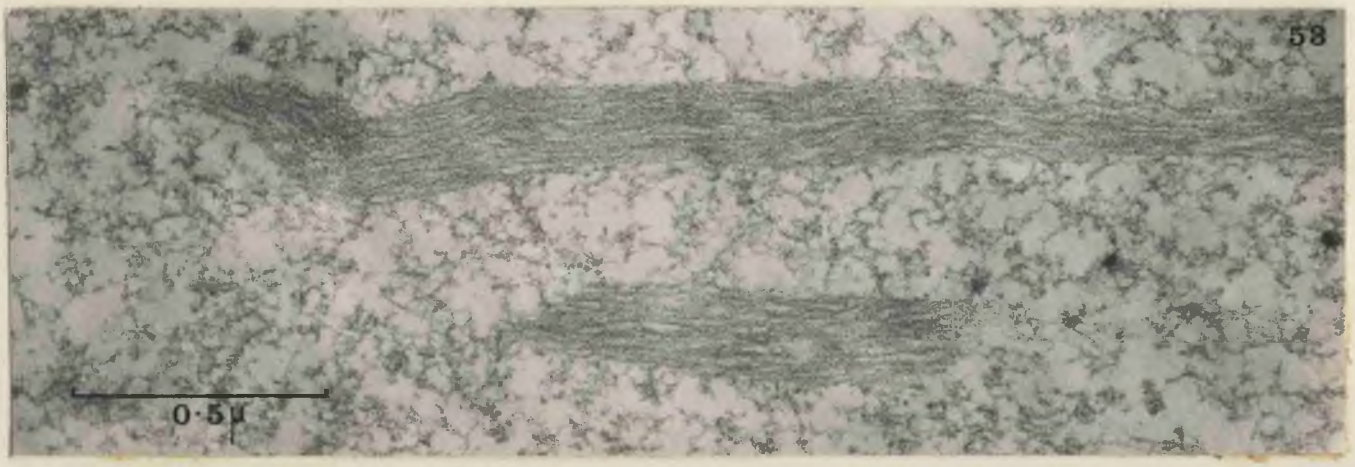

53
0·5μ

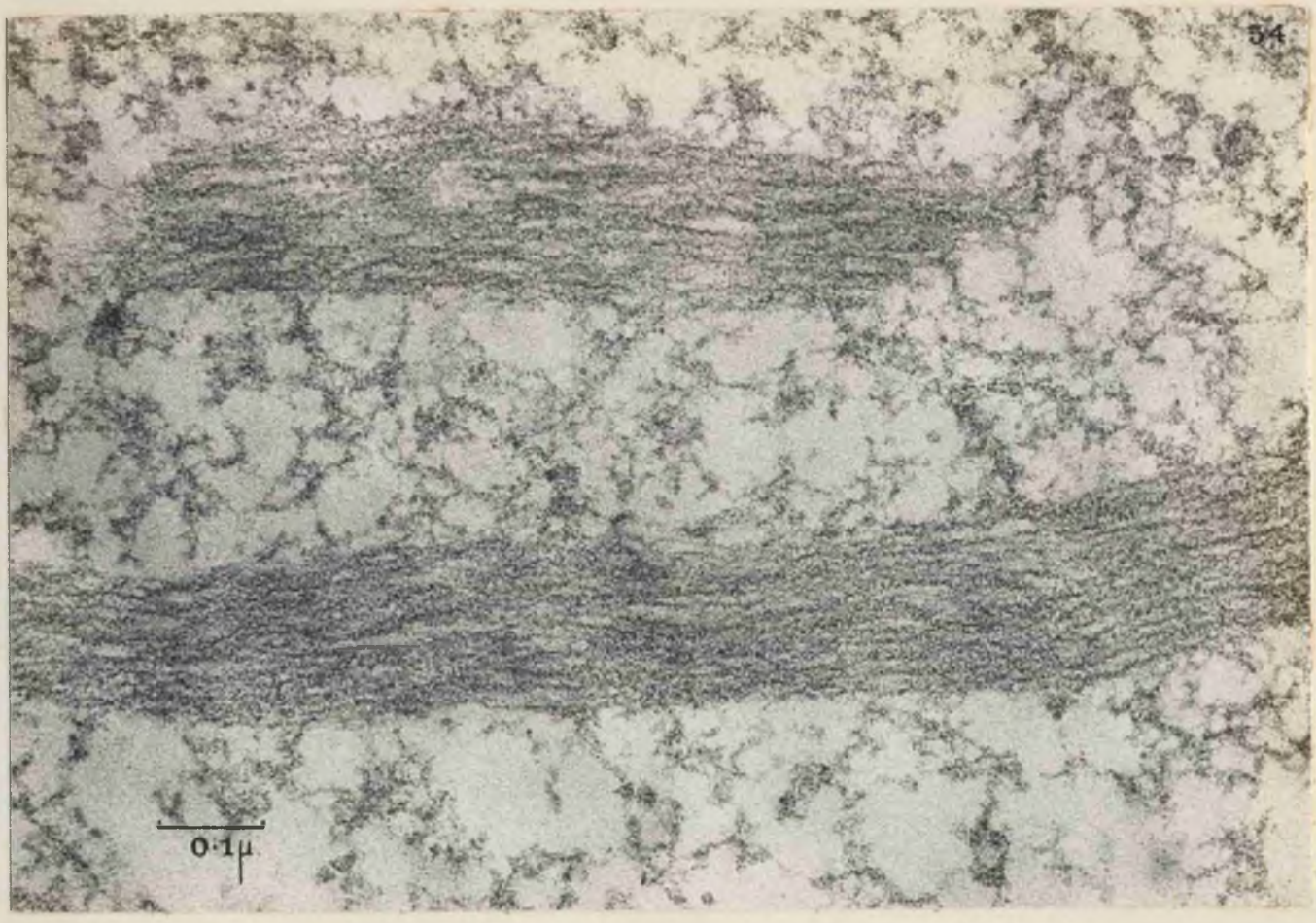
PLATE IX

Electron microscopy: Lamellar bodies.

Fig.54: Part of fig. 53. The continuity between nuclear sap and the lamellar body is very apparent in the centre of the picture. x 120,000

Fig.55: A longitudinal section and a transverse section through small lamellar bodies. Again continuity between these bodies and the nucleoproteins in the nuclear sap is evident. x 60,000

Fig.56: A lamellar body cut in transverse section clearly showing the lamellar structure. x 120,000


54
0·1μ


55
0·5μ

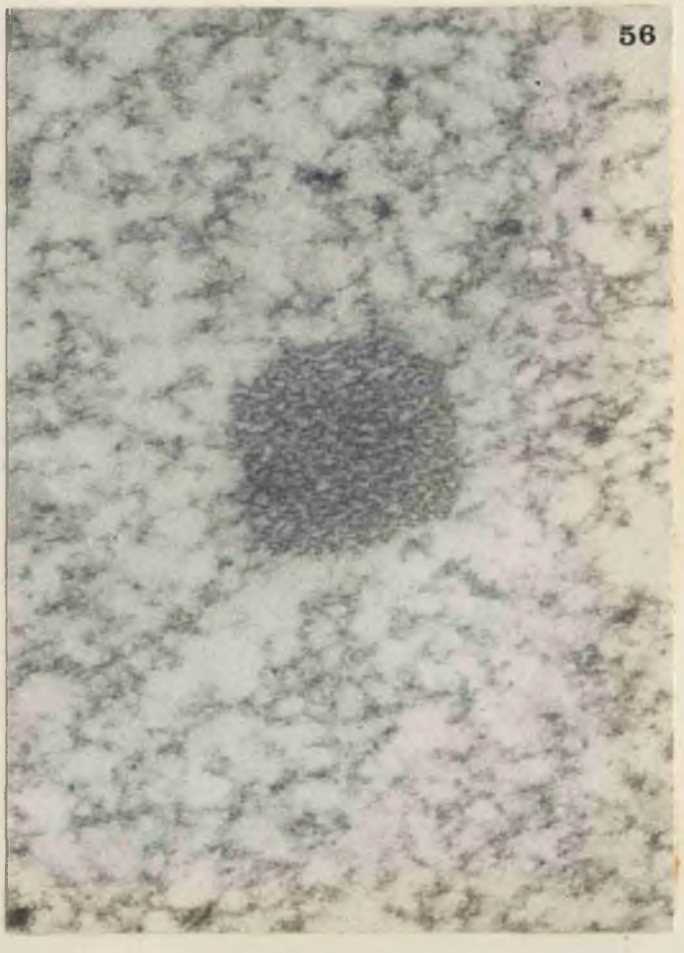
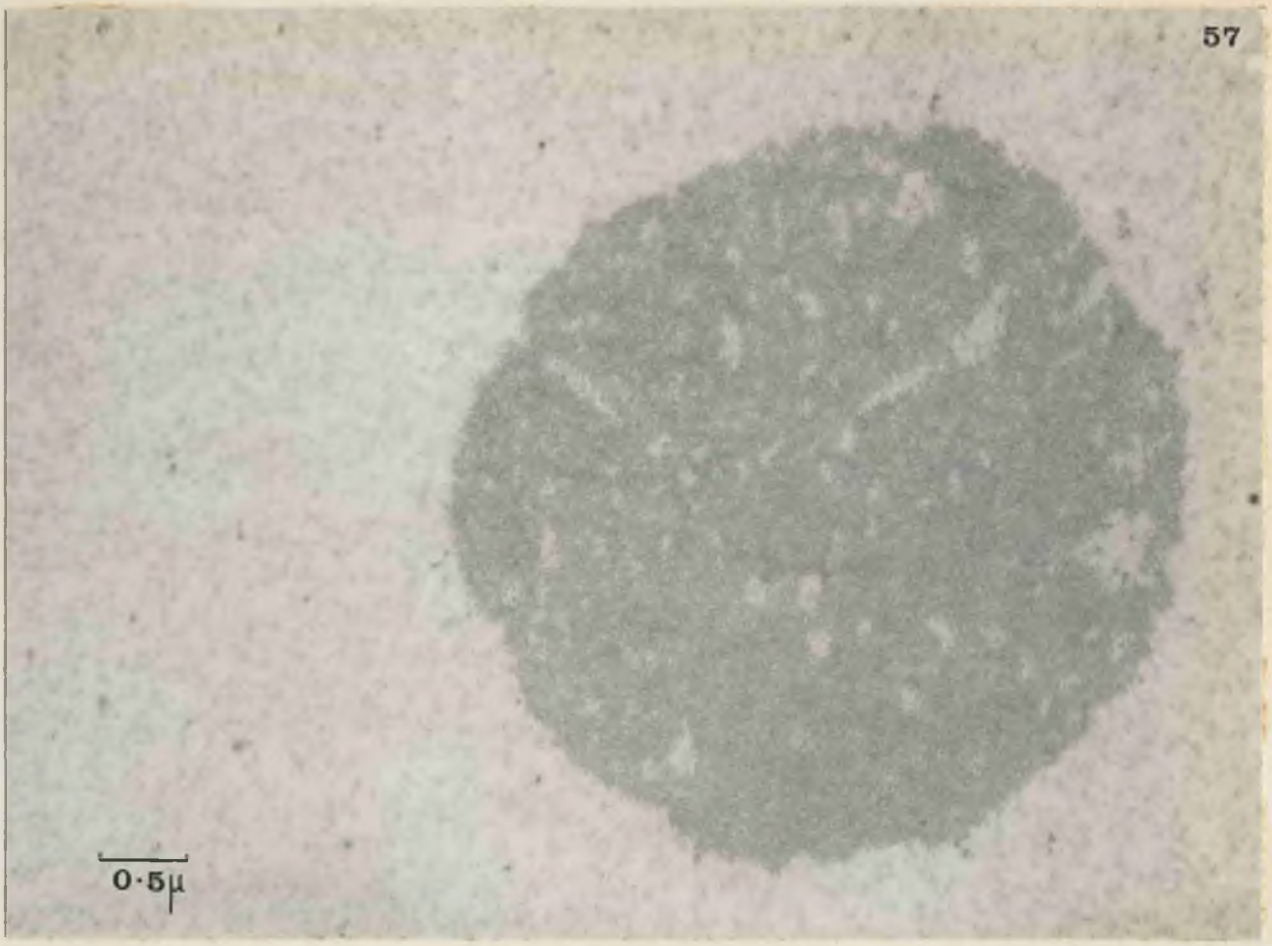

56

PLATE X

Electron microscopy: peripheral nucleoli, one day after Actinomycin D treatment.

Figs.57&58: From oocytes 0.8mm. and 1.0mm. diameter. Note the considerable vacuolation of these nucleoli. At this stage the nucleoli have regained some of their size and some of the granular component. x 20,000


57
0·5μ

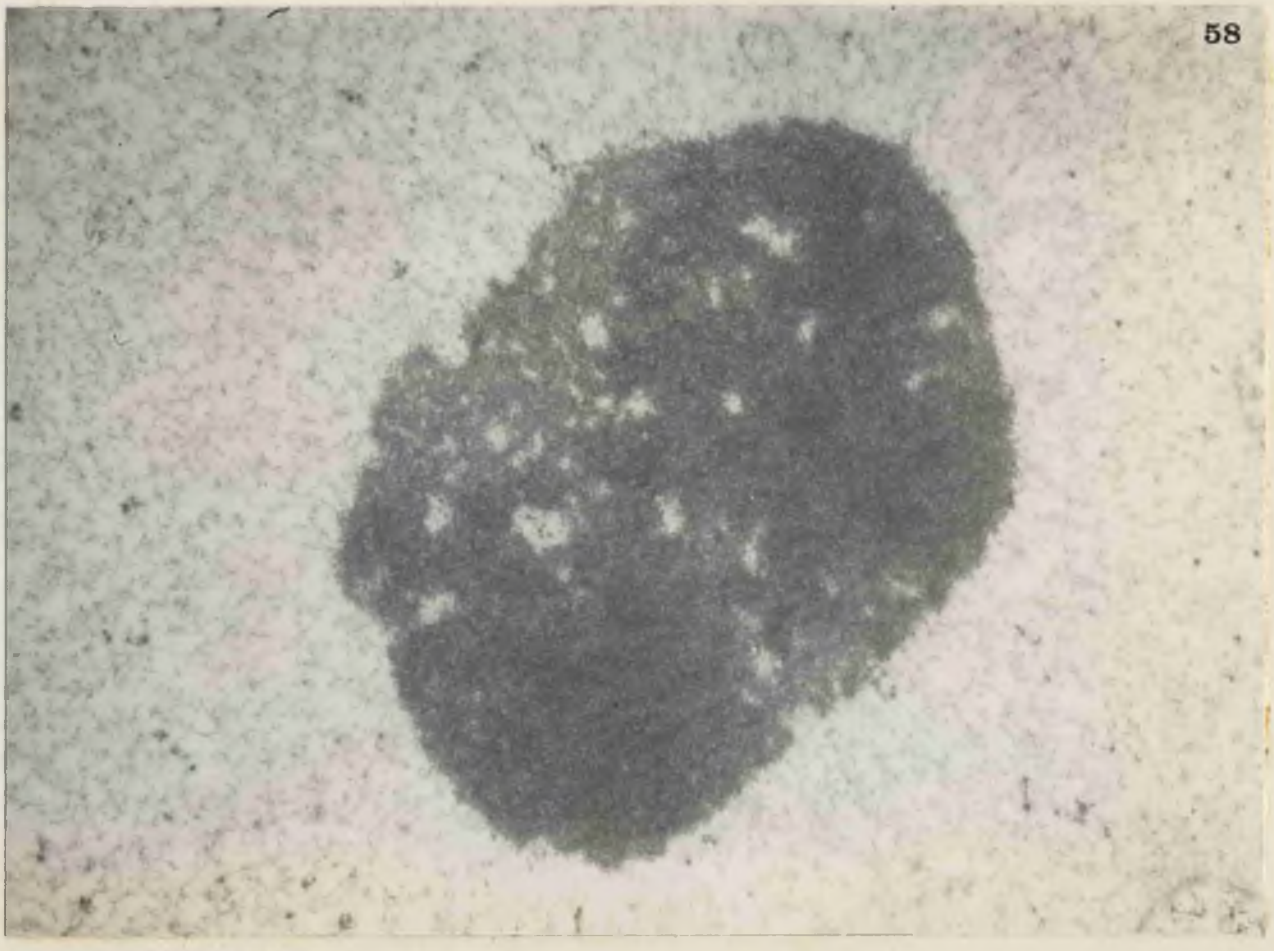

58

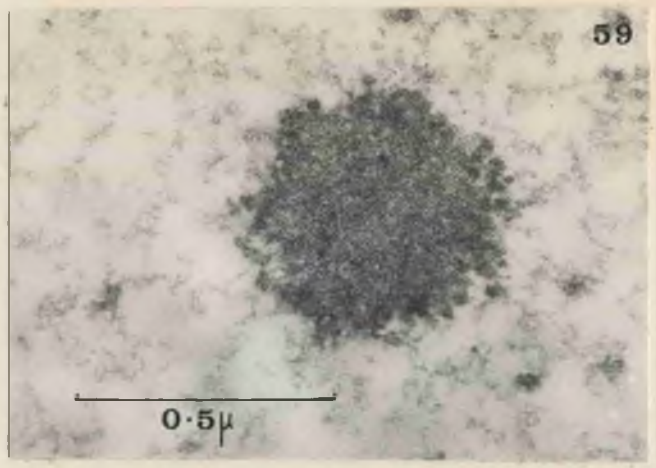
PLATE XI

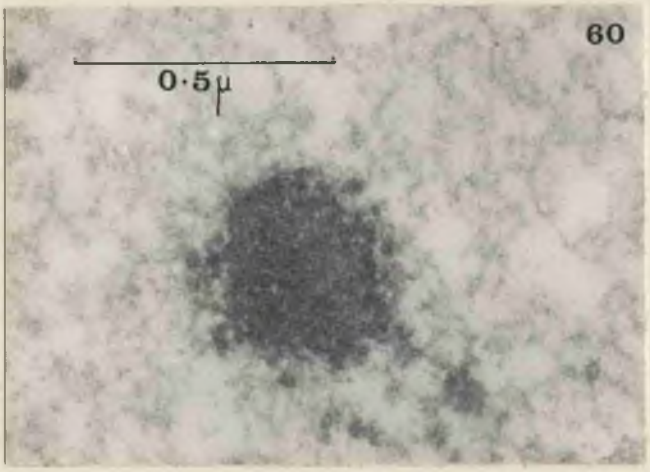
Electron microscopy; peripheral nucleoli and micronucleoli.

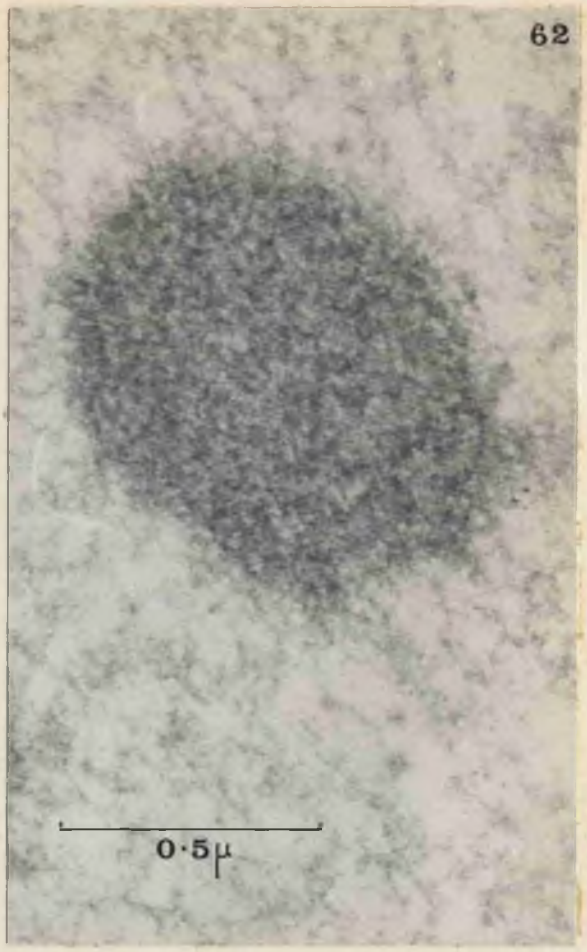
Figs.59&60: Micronucleoli 1 day after Actinomycin D treatment. The granular aggregates are greatly reduced in number. x 60,000

Fig.61: Peripheral nucleolus 2 days after Actinomycin D treatment. There is still some evidence of vacuolation but the components of the nucleolus are essentially normal. x 20,000

Fig.62: Micronucleolus 2 days after Actinomycin D treatment. The granular component is similar to that found in untreated oocytes. For comparison see fig. 44 & 45. x 60,000


59
0·5μ


60
0·5μ


62
0·5μ

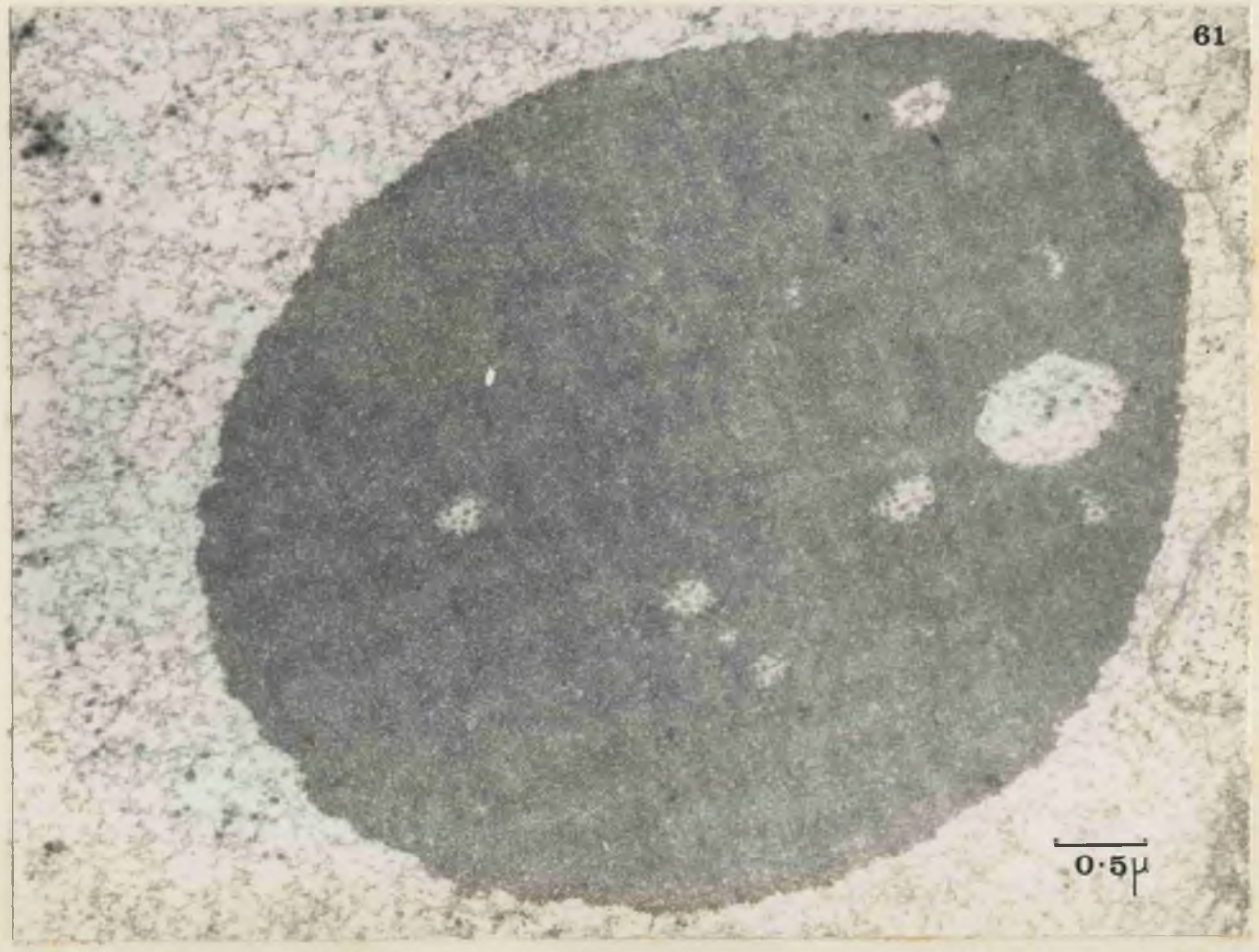

61
0·5μ

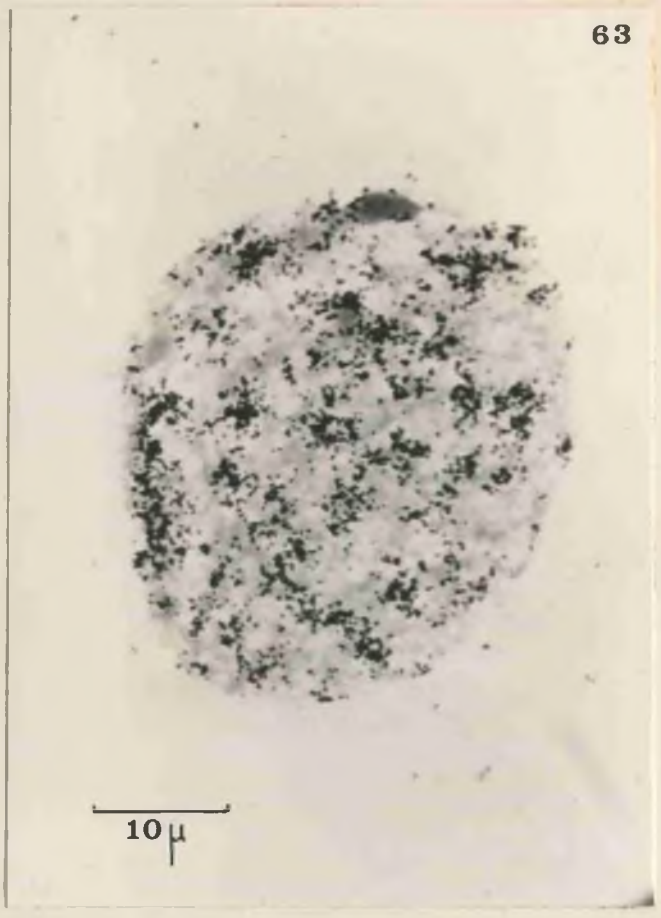
PLATE XII

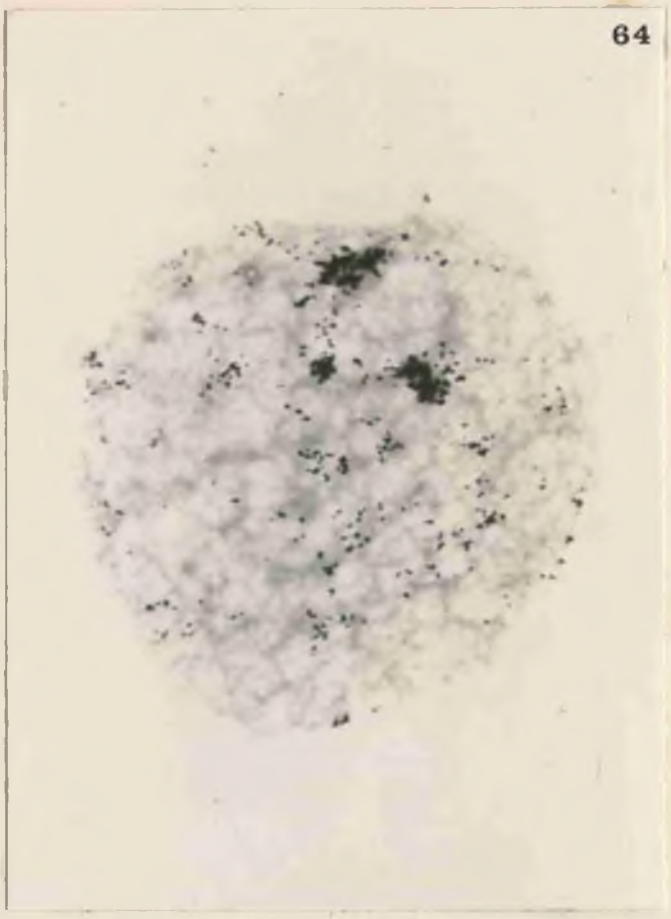
Testis squashes: Batrachoseps attenuatus meiotic prophase

Fig.63: Spermatocyte from a testis fixed 4h. after H^3-thymidine injection. The cell is in early 'S' phase. Note the absence of silver grains over the chromocentre at the top of the nucleus. Exposure time 21 days.

Fig.64&65: As fig. 63. These spermatocytes were labelled in late 'S' phase. Note the localisation of silver grains over the chromocentre (s). This late labelling pattern is readily identifiable throughout prophase. Exposure time 21 days.

Fig.66: From a testis fixed 15 days after H^3-thymidine injection. Synapsis is just commencing and is characterised by the segregation of chromosome ends at the opposite pole of the nucleus to the chromocentre. Exposure time 25 days.


63
10 μ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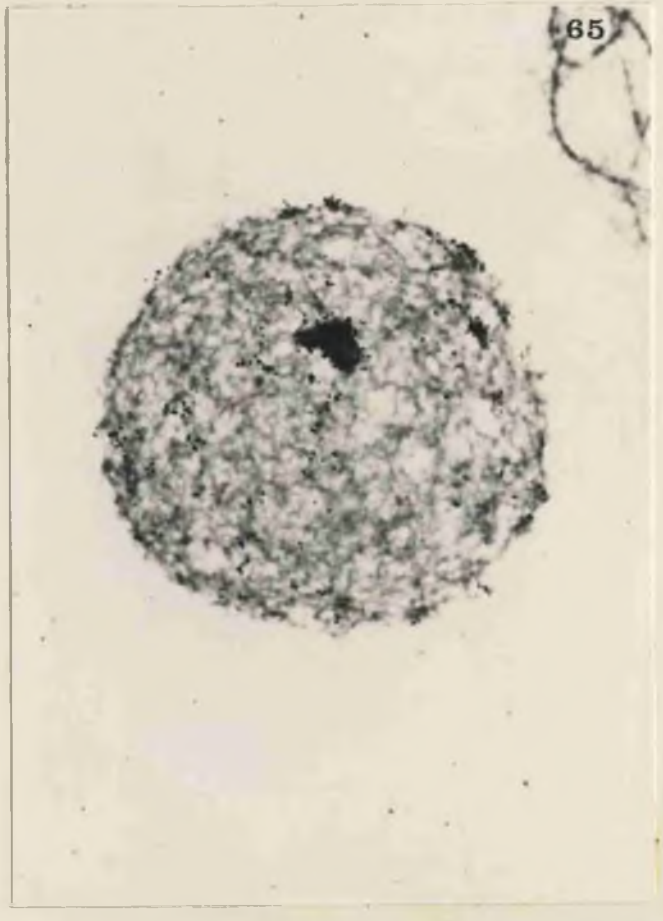

65


66

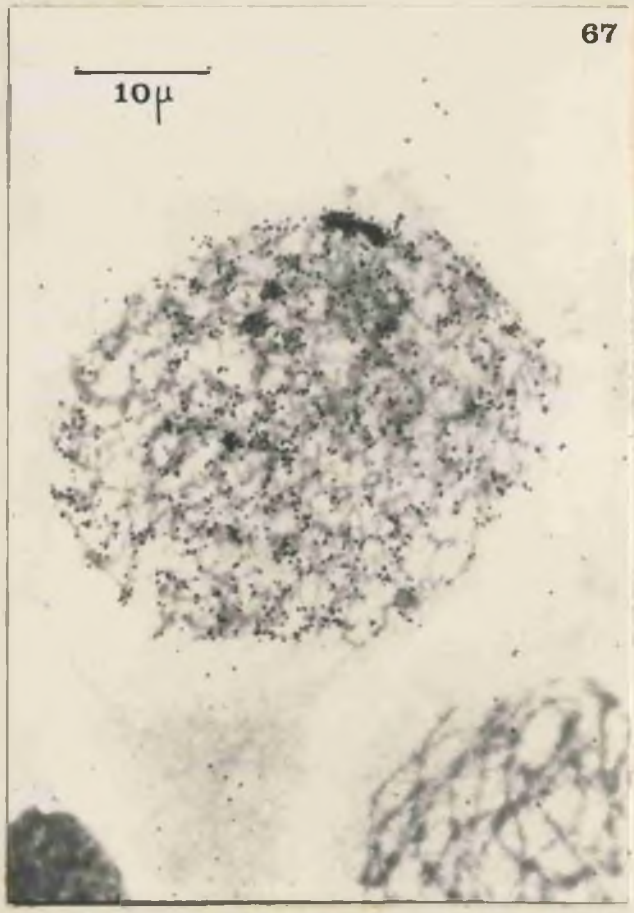
PLATE XIII

Testis squashes: Batrachoseps attenuatus meiotic prophase

Fig.67: As fig. 65. Exposure time 25 days.

Fig.68: From a testis fixed 39 days after H^3-thymidine injection. Synapsis is almost complete. Note that the chromosomes are unpaired in the regions adjacent to the chromocentres. Exposure time 26 days.

Figs.69&70: Two full, unlabelled, pachytenes from the same testis as fig. 68. The chromocentre has divided into 8 and 7 parts respectively.


67
10μ


68


69


70

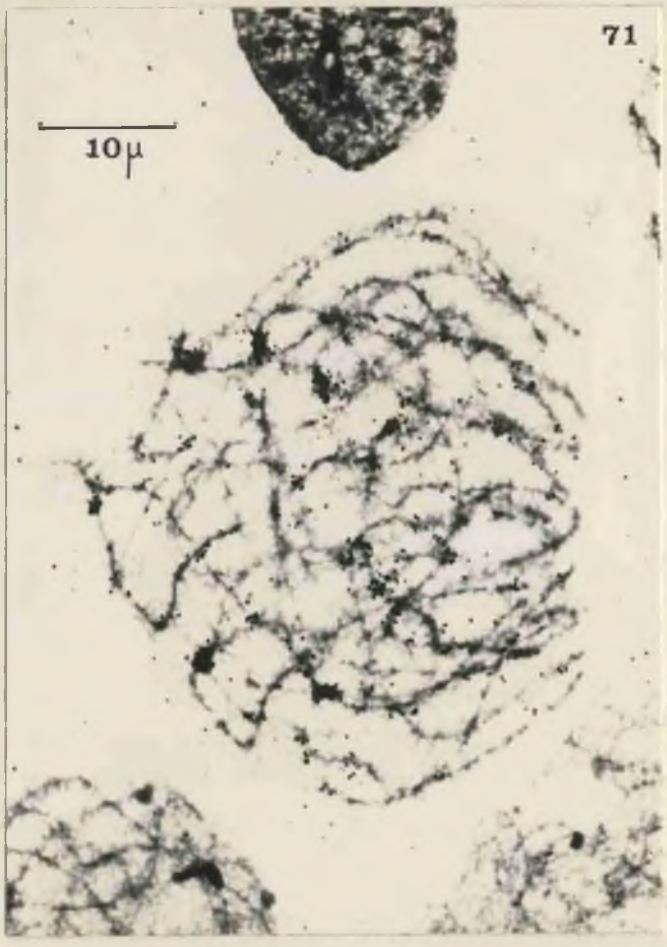
PLATE XIV

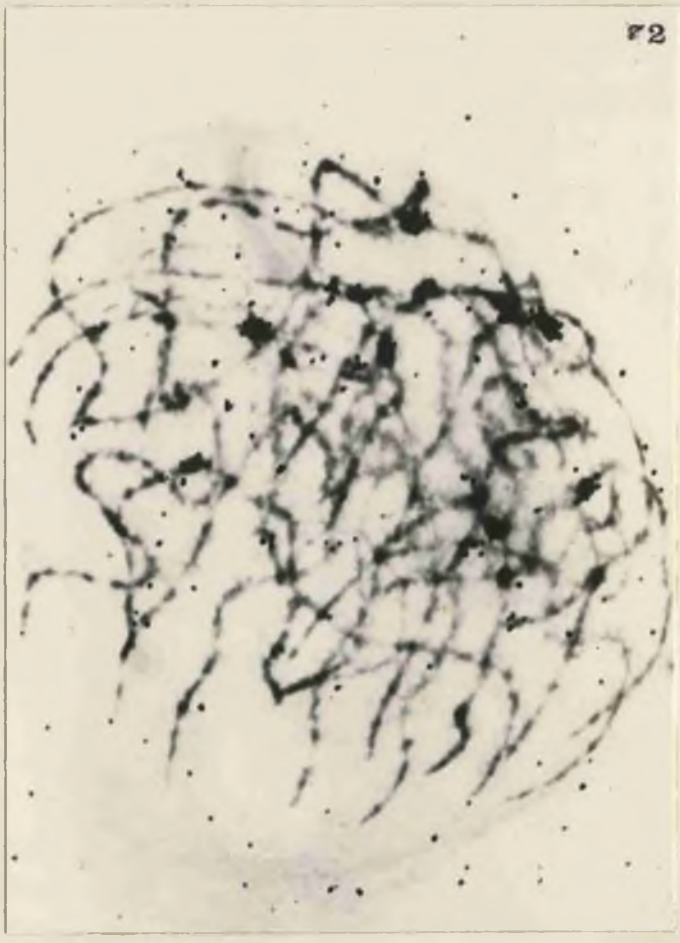
Testis squashes: Batrachoseps attenuatus meiotic prophase

Figs.71&72: Full pachytenes from the same testis as figs. 68 - 70. These chromosomes exhibit the characteristic late labelling pattern. Note the 8 chromocentres in fig. 71. Exposure time 26 days.

Fig.73: A labelled early diplotene from a testis fixed 83 days after H^3-thymidine injection. Exposure time 45 days.

Fig.74: A later diplotene stage from the same testis that gave fig. 73.


71
10μ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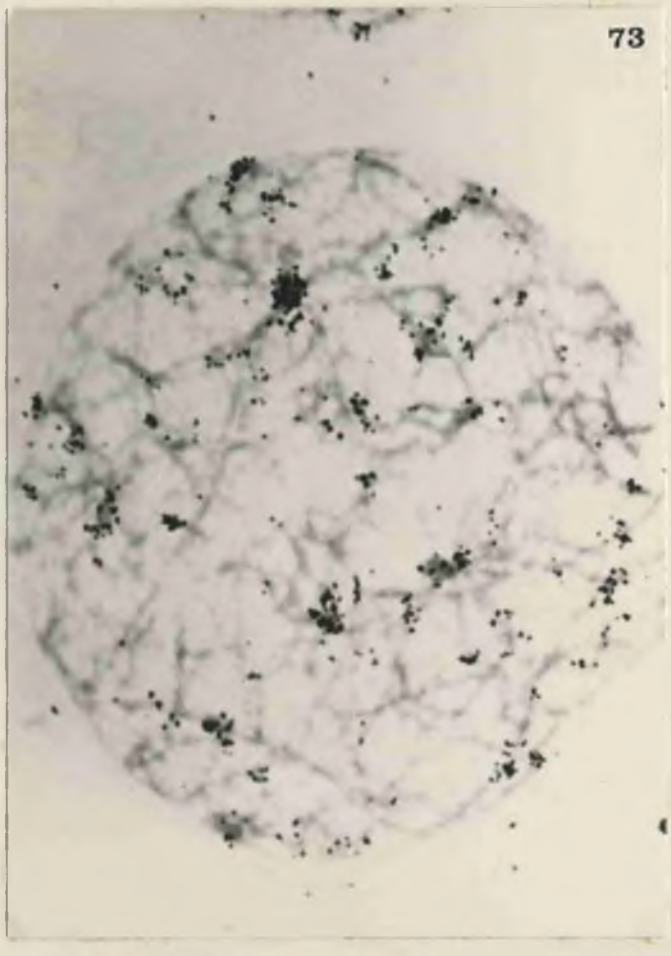

73


74

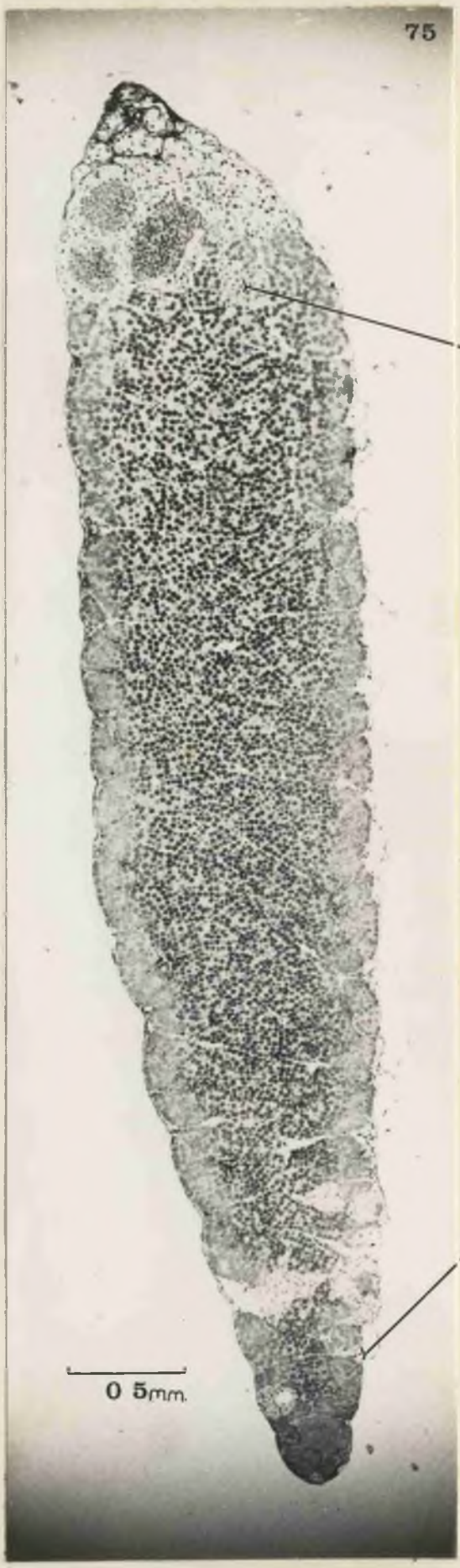
PLATE XV

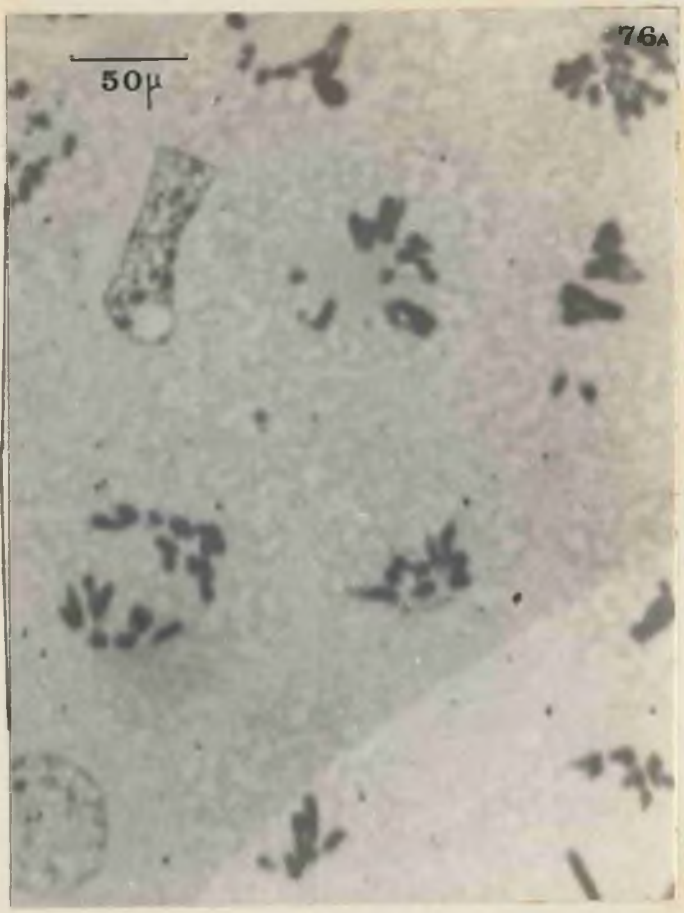
Testis section: Batrachoseps attenuatus.

Fig.75: A longitudinal section of a testis fixed 8 days after arrival in the laboratory. This is from animal Bat. 7 + 6 days and the testis contains a full range of spermatocyte stages as the accompanying enlargements illustrate.

Fig.76a: Division stages in the testis.

Fig.76b: 'S' phase cells in the same testis.


75
0 5mm.


50μ
76A

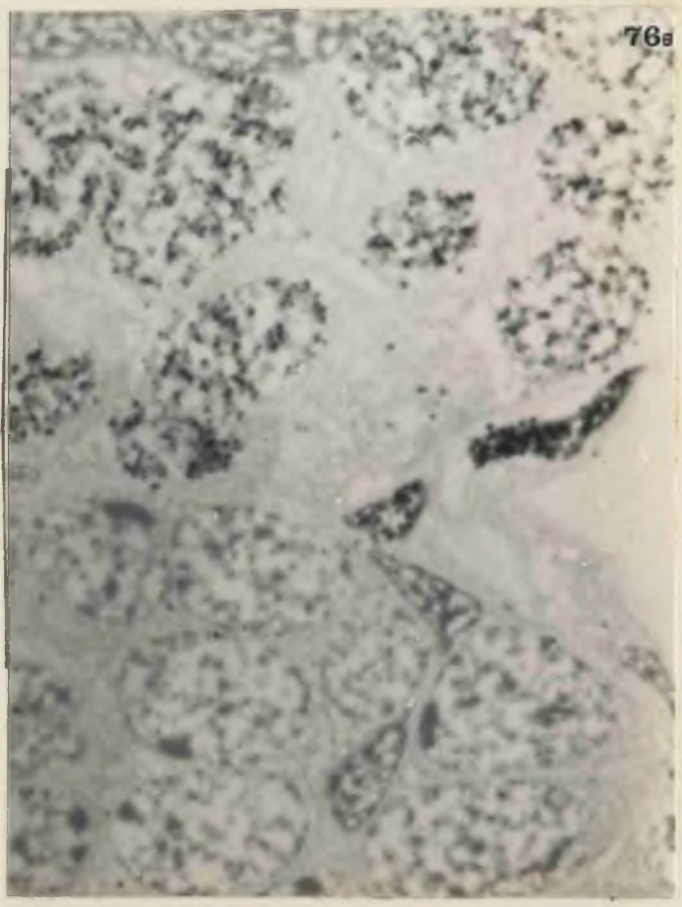

76B

PLATE XVI

Testis squashes: Bufo bufo meiotic prophase.

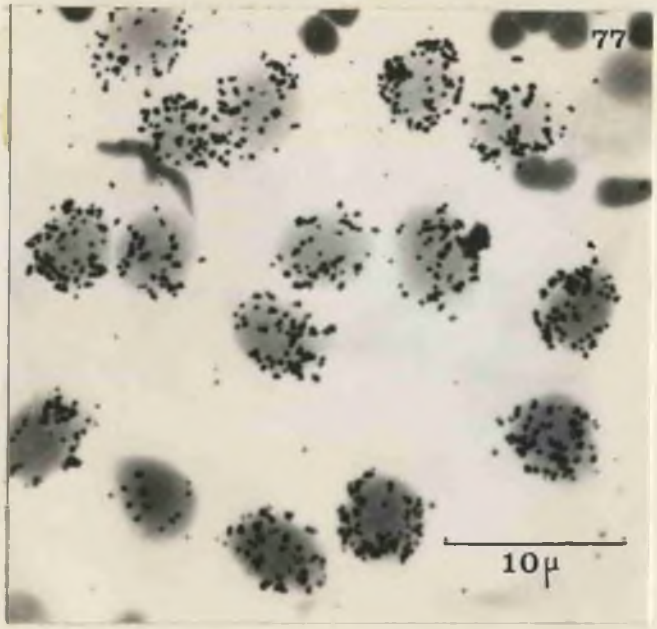
Fig.77: Labelled leptotene cells from a testis fixed 4h. after H^3-thymidine injection. No late labelling pattern is evident. Exposure time 27 days.

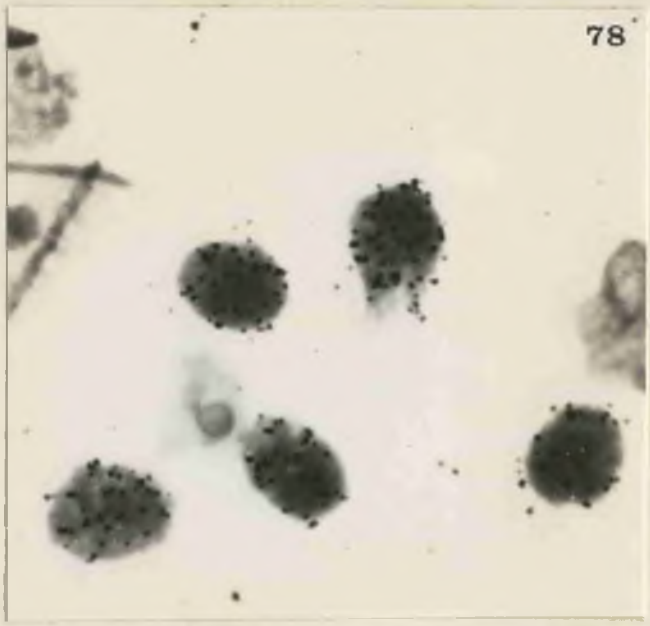
Figs.78&79: Nuclei clearly in early synapsis are indicated by arrows. Testis fixed 6 days after H^3-thymidine injection. Exposure time 27 days.

Fig.80: Nuclei in late synapsis from a tesits fixed 17 days after H^3-thymidine injection. Exposure time 27 days.

Fig.81: The nucleus at the top of this picture is probably in very early pachytene. Two well advanced synizetic knots, representing late pachytenes, can be seen at the bottom left. Testis fixed 20 days after injection. Exposure time 39 days.

Fig.82: This late synizetic knot contains diplotene chromosomes. Testis fixed 27 days after injection. Exposure time 39 days.


77
10μ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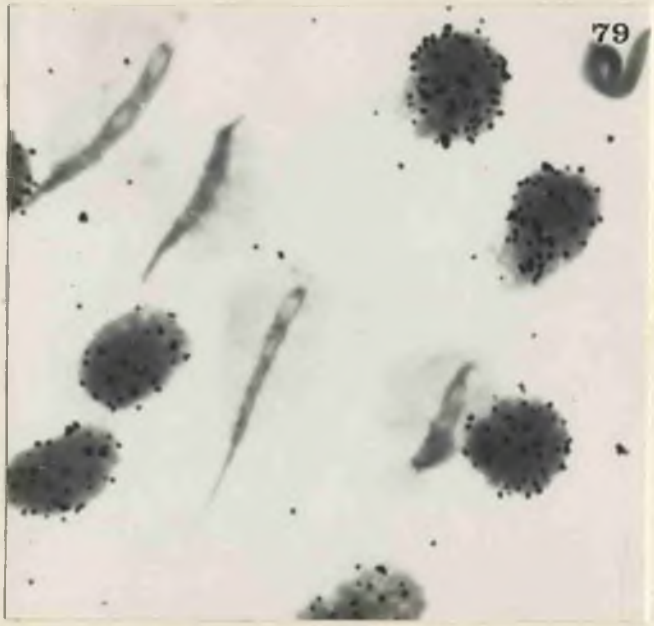
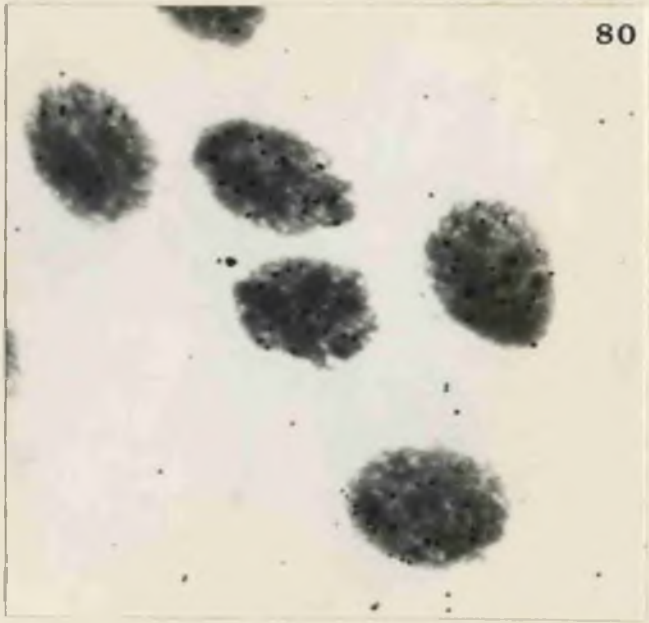

79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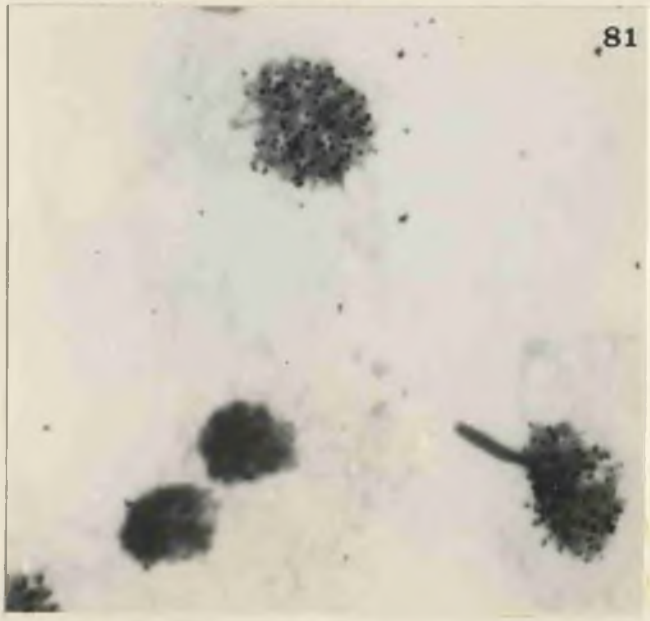
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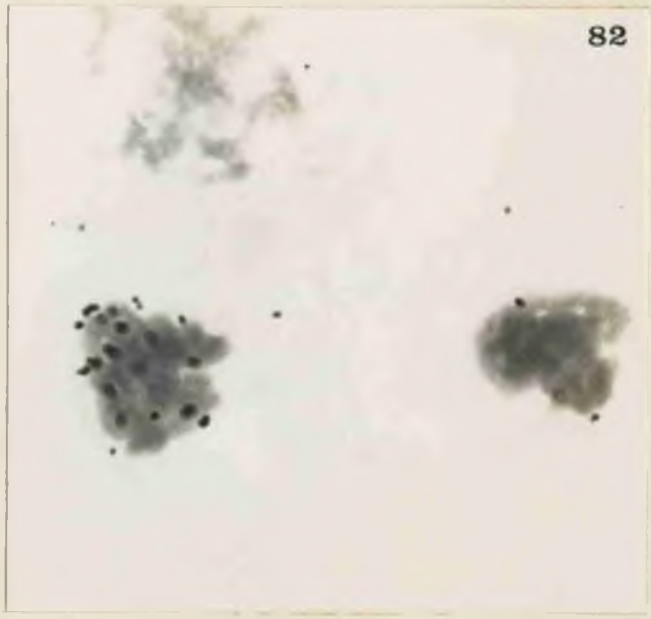

82

PLATE XVII

Testis squashes: Bufo bufo meiotic metaphase and Xenopus laevis meiotic prophase.

Bufo bufo

Fig.83: Two labelled metaphases. Testis fixed 29 days after H^3-thymidine injection. Exposure time 39 days.

Xenopus laevis

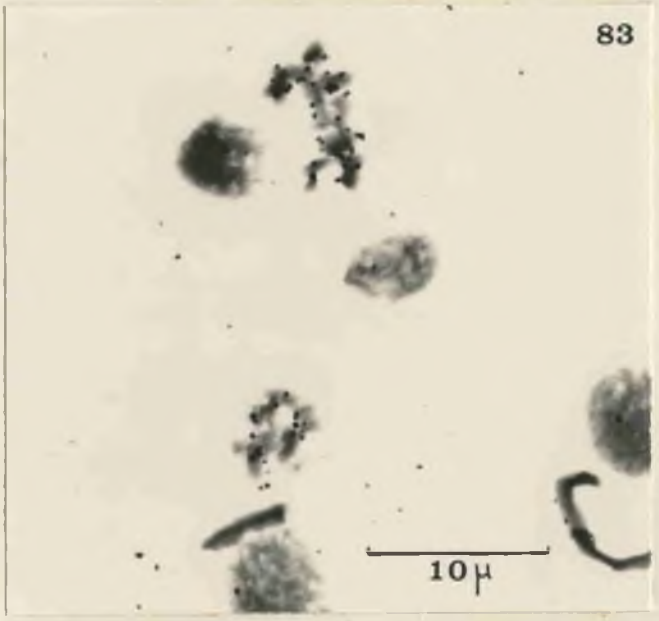
Fig.84: Labelled leptotenes from a testis fixed 4h. after injection of H^3-thymidine. Exposure time 27 days.

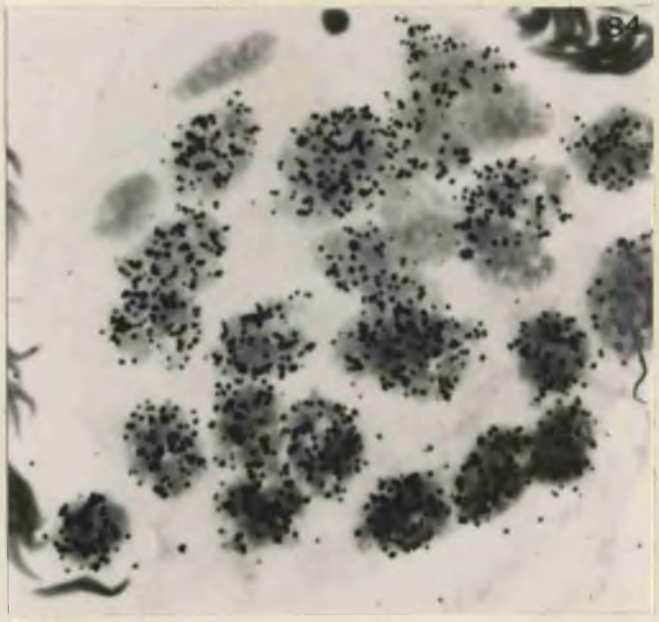
Fig.85: Nuclei in early synapsis are indicated by arrows. Testis fixed 6 days after H^3-thymidine injection. Exposure time 27 days.

Fig.86: Labelled nuclei in pachytene. Testis fixed 18 days after H^3-thymidine injection. Exposure time 46 days.

Fig.87: Severely squashed synizetic knots yielding chromosomes in diplotene. Testis fixed 42 days after H^3-thymidine injection. Exposure time 46 days.

Fig.88: Synizetic knots and metaphase plates from a testis fixed 45 days after H^3-thymidine injections. No silver grains are found over these nuclei after a 46 day exposure.


83
10μ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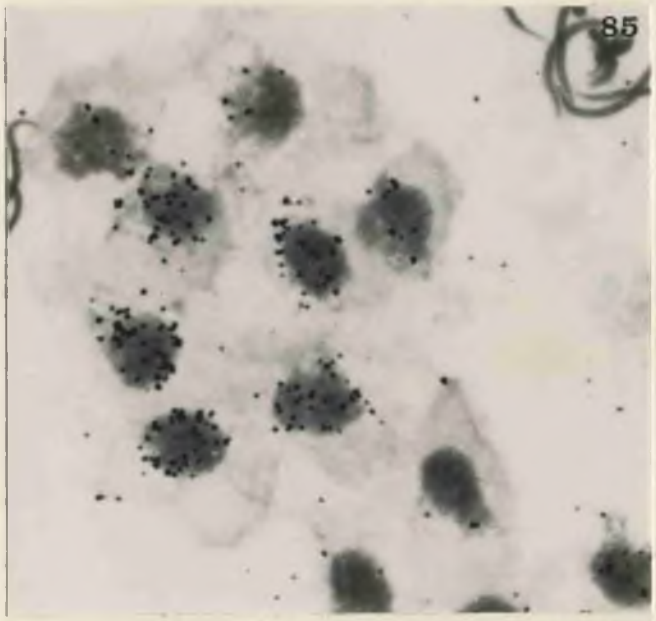
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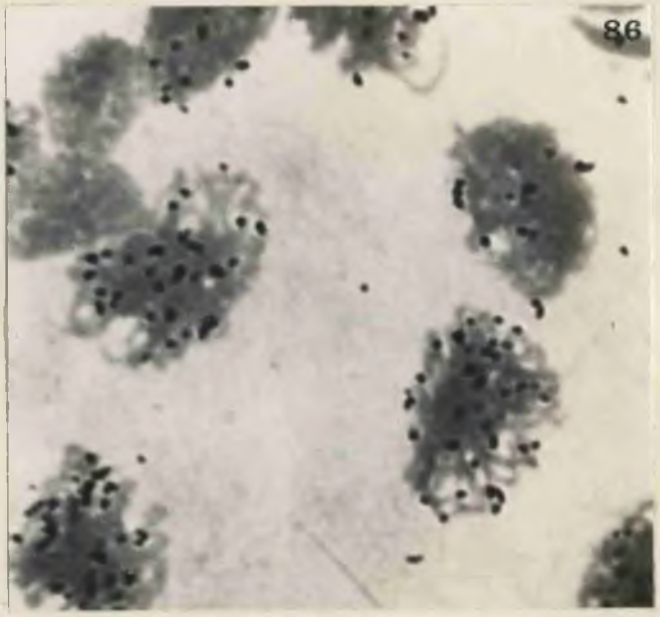
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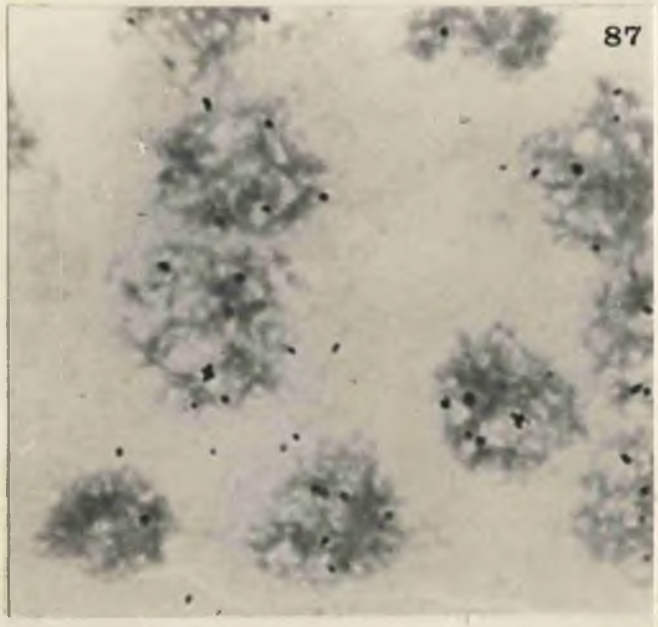
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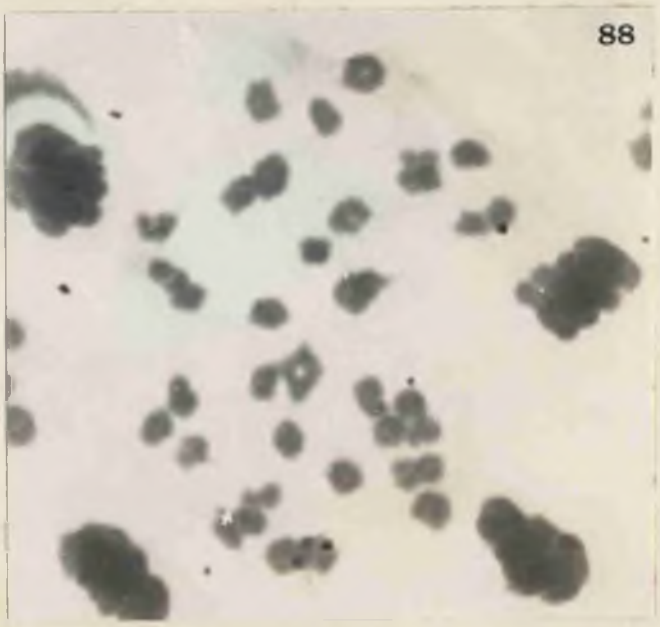
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